



(51) International Patent Classification:

A61K 39/00 (2006.01) A61P 7/12 (2006.01)
A61K 47/68 (2017.01) A61P 9/10 (2006.01)

(21) International Application Number:

PCT/US2020/052524

(22) International Filing Date:

24 September 2020 (24.09.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

62/905,759 25 September 2019 (25.09.2019) US

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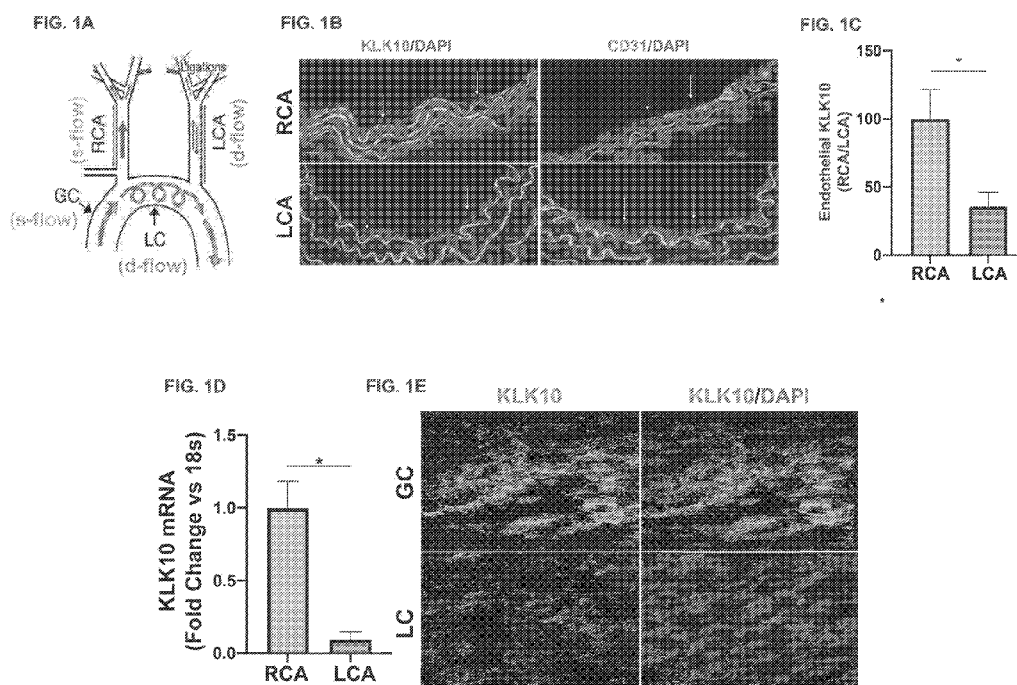
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW,

(54) Title: USE OF KLK10 AND ENGINEERED DERIVATIZATIONS THEREOF



(57) Abstract: Methods are disclosed herein for treating atherosclerosis and/or decreasing arterial endothelial inflammation in a subject. These methods include administering to the subject a therapeutically effective amount of a Kallikrein Related Peptidase 10 (KLK10) protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein. In some embodiments, the fusion protein is a KLK10 protein fused to an Fc domain.

SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

- (84) Designated States** (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
- *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

- *with international search report (Art. 21(3))*
- *with sequence listing part of description (Rule 5.2(a))*

USE OF KLK10 AND ENGINEERED DERIVATIZATIONS THEREOF**STATEMENT OF GOVERNMENT SUPPORT**

This invention was made with government support under Grant No. HL139757 awarded by
5 the National Institutes of Health. The government has certain rights in the invention.

CROSS REFERENCE TO RELATED APPLICATION

This claims the benefit of U.S. Provisional Application No. 62/905,759 filed September 25,
10 2019, which is incorporated herein by reference.

FIELD OF THE DISCLOSURE

This relates to the field of atherosclerosis, specifically to the use of a Kallikrein Related
Peptidase 10 (KLK10) protein, a fusion protein comprising the KLK10 protein, or a nucleic acid
molecule encoding the KLK10 protein or the fusion protein to treat atherosclerosis.

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PARTIES TO JOINT RESEARCH AGREEMENT

This invention was made under a Sponsored Research Agreement between Emory
University and Celltrion, Inc., which was executed prior to the filing date of U.S. Provisional
Application No. 62/905,759.

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BACKGROUND

Atherosclerotic cardiovascular disease is the leading cause of death in the U.S. and is
becoming the leading killer in developing countries. The lifetime risk of developing coronary heart
disease is 1 in 2 for men and 1 in 3 for women. Cardiovascular disease has ranked highest among
25 all disease categories in hospital discharges; 16 million people in the US have coronary heart
disease and 7 million have a history of stroke. Each year, an estimated 785,000 Americans will
have a first coronary heart disease event and 610,000 a first stroke. Between 2010 and 2030, total
direct medical costs of cardiovascular disease are projected to triple, from \$273 billion to \$818
billion. There is a need for methods of treating atherosclerosis and for methods of treating and
30 inhibiting atherosclerotic cardiovascular disease and related disorders.

SUMMARY OF THE DISCLOSURE

Disclosed herein is a method of treating atherosclerosis in a subject. The method includes
administering to the subject a therapeutically effective amount of a Kallikrein Related Peptidase 10

(KLK10) protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, thereby treating the atherosclerosis in the subject.

In another embodiment, a method is disclosed for decreasing arterial endothelial inflammation in a subject. In some examples, the method includes selecting a subject with atherosclerosis, and administering to the subject a therapeutically effective amount of a KLK10 protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, thereby decreasing arterial endothelial inflammation in the subject.

In certain embodiments of the disclosed methods, the subject has a stroke, peripheral artery disease, or myocardial infarction. In further embodiments, the fusion protein comprising the KLK10 protein further includes an Fc domain.

Also disclosed are compositions comprising a KLK10 protein that includes an amino acid sequence at least 90% identical to SEQ ID NO: 1 or SEQ ID NO: 2, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, for use in any of the methods disclosed herein.

The foregoing and other features and advantages of the invention will become more apparent from the following detailed description of several embodiments which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE FIGURES

FIGS. 1A-1J. KLK10 expression is suppressed by disturbed flow (d-flow) and elevated by stable flow (s-flow) in endothelial cells in vitro and in vivo. (A-F) Depiction of partial carotid ligation (PCL) surgery and flow-sensitive regions in the aortic arch: right carotid artery (RCA; s-flow), left carotid artery (LCA; d-flow), greater curvature (GC: s-flow), and lesser curvature (LC; d-flow). Two days following the PCL of C57BL/6 mice, RCA and LCA were collected to prepare for frozen section imaging (B-C) and (D) endothelial-enriched RNA preparation used for KLK10 qPCR. Shown are confocal images of immunostaining with anti-KLK10 or anti-CD31 antibodies (B) and its quantification (C). Scale bar=200 μ m. Arrows indicate endothelial cells. (E) Confocal images of the en-face immunostaining of LC and GC with anti-KLK10 antibody are shown. Scale bar=20 μ m. (F) shows the quantification of endothelial KLK10 in (E). Paired two-tailed t-test. Mean $\pm$ SEM. * $P\leq 0.05$, n=4. (G-J) HAECs subjected to 24h of unidirectional laminar shear (LS, 15 dynes/cm²) or oscillatory shear (OS, ± 5 dynes/cm² at 1Hz) were used to measure expression of KLK10 mRNA by qPCR(G), KLK10 protein in cell lysates by western blot (H, I) and (J) KLK10

protein secreted to the conditioned media by ELISA. Paired two-tailed t-Test. Mean±SEM.

*P≤0.05, n=6-9.

FIGS. 2A-2K. KLK10 inhibits inflammation in endothelial cells *in vitro* and *in vivo*.

(A) THP1 monocyte adhesion assay was carried out in HAECs treated with plasmids expressing
 5 KLK10 (KLK10-p) or GFP (GFP-p) with or without TNFα (5ng for 4h). (B) THP1 monocyte
 adhesion assay was carried out in HAECs treated with TNFα with or without rKLK10 at 0.1-10
 ng/mL or heat-inactivated (HI-10) rKLK10 at 10 ng/mL. (C, D) Inflammatory marker VCAM1
 and ICAM1 expression were assessed by qPCR (C,D) or Western blot (E-G) in HAECs treated
 with rKLK10 at 0.1-10 ng/mL. (H,I) Monocyte adhesion assay was conducted on HAECs
 10 subjected to 24h of either LS or OS with either (H) rKLK10 (100 ng/mL) or (I) KLK10 siRNA
 (50nM) for KLK10 or a non-targeting siRNA control. Mean±SEM, n=3-6. *p<.05. (J) C57BL/6
 mice were injected with rKLK10 (0.6 mg/kg) or a vehicle control on days 0, 2, and 4, and
 sacrificed on day 5. The aortic arches were *en-face* immunostained using an anti-VCAM1
 antibody. Shown in (J) are confocal images of endothelial KLK10 in LC and GC with DAPI for
 15 nuclear staining and auto-fluorescence (matrix) and (K) its quantification. Scale bar=10μm. One-
 way ANOVA with Bonferroni correction for multiple comparisons. Mean±SEM n=6.*P≤0.05.

FIGS. 3A-3D. KLK10 protects endothelial permeability against thrombin and OS.

(A) Confluent HAECs grown on biotinylated gelatin was treated with rKLK10 (10 ng/mL) or
 vehicle for 20h followed by (A-B) thrombin (5 U/mL for 30 minutes) or (C-E) OS were used to
 20 measure FITC-avidin binding. Scale bar=100μm. (A, C) are confocal images and (B, D) are their
 quantifications. Data was pooled from at least three independent experiments, n=4-6. One-way
 ANOVA with Bonferroni correction (B) or paired two-tailed T-Test (D). Mean±SEM.*P≤0.05.

FIGS. 4A-4I. Treatment with rKLK10 inhibits atherosclerosis development in *ApoE*^{-/-}

mice. (A) *ApoE*^{-/-} were subjected to partial carotid ligation and high fat diet feeding. The mice
 25 received either rKLK10 (0.6 mg/kg) or vehicle injection every three days for the duration of three
 weeks. LCA (*d-flow*) show plaque development, which was reduced by rKLK10 as shown by
 dissection microscopy (A). LCA and RCA (*s-flow*) tissue were isolated and frozen sections were
 stained with (B) H&E and (C) for CD68 in LCA. DAPI. Scale bar low mag=250μm, high
 mag=50μm. (D) Atherosclerotic burden shown in (B) and (E) CD68 macrophage accumulation was
 30 quantified. (F-I) Plasma lipid analysis of (F) total cholesterol, (G) LDL cholesterol, (H) HDL
 cholesterol, or (I) triglycerides showed no effect of rKLK10 compared to control. Paired two-tailed
 t-test. Mean±SEM. n=6. *P≤0.05.

FIGS. 5A-5N. Ultrasound-mediated expression using KLK10 plasmid inhibits

atherosclerosis development. Following partial ligation, *ApoE*^{-/-} mice were injected with plasmids

expressing KLK10-luciferase or luciferase along with microbubbles in the hind-limb, then sonoporated with ultrasound (0.35 W/cm^2 for 1 minute), and fed a high-fat diet for 3 weeks. The plasmids plus microbubble injection and sonoporations were repeated at day 10. (A) Bioluminescent imaging (shown in greyscale intensity scale) of *the* mice with luciferin show abundant luciferase expression at day 7. Three weeks later, mice were sacrificed, LCAs and RCAs and aortic sinus were excised. (B) Gross plaque images of excised carotid arteries (B, C) and the H&E staining of the aortic sinus (D,E) are shown. (C, E) show quantification of (B, D), respectively. (D) Scale bar low mag= $250\mu\text{m}$, high mag= $50\mu\text{m}$. Mean $\pm$ SEM, * $P\leq 0.05$, t-test, $n=11-12$. (F, G) Frozen sections from the RCA and LCA were further stained with anti-KLK10. DAPI staining denotes nuclear staining and Auto-fluorescence elastin lamina is also shown. Arrows indicate the endothelial layer. Scale bar= $20\mu\text{m}$ (G) Quantification of endothelial KLK10 fluorescence from F. Mean $\pm$ SEM, $n=4-5$. t-test, * $P<0.05$. (H) Western blot assessing KLK10 expression in lung tissue from mice injected with control luciferase plasmid or KLK10 plasmid. GAPDH was used as a loading control. (I) Quantification of (H). Paired two-tailed t-test. Mean $\pm$ SEM. $n=5$, * $P\leq 0.05$. (J-N) Plasma lipid analysis of (J) total cholesterol, (K) triglycerides, (L) HDL cholesterol, (M) LDL cholesterol, or (N) non-HDL cholesterol. Paired two-tailed t-test. Mean $\pm$ SEM. $n=5$, * $P\leq 0.05$.

FIGS. 6A-6G. KLK10 inhibits endothelial inflammation in a PAR1/2-dependent manner, but without the direct cleavage. (A) THP1 monocyte adhesion assay was conducted in HUVECs subjected to OS ($\pm 5 \text{ dynes/cm}^2$) for 24h to induce endothelial inflammation in the presence of rKLK10 as well as the (A) PAR1 and PAR2 inhibitors or (B) siRNAs for PAR1, PAR2 or non-targeting control (Ctrl) for 24h. Paired two-tailed t-test. Mean $\pm$ SEM. $n=6$, * $P\leq 0.05$. (C) PAR cleavage assay in which HAECs were transfected with PAR1-AP or PAR2-AP plasmids and treated with rKLK10 (100 ng/mL), thrombin (5 U/mL) or trypsin (5 U/mL). Conditioned media were assayed for secreted alkaline phosphatase activity. (D) PAR cleavage assay in which HAECs were co-transfected with PAR1-AP or PAR2-AP plasmids and KLK10 plasmids. Conditioned media were assayed for alkaline phosphatase activity. Paired two tailed t-test. Mean $\pm$ SEM. $n=6$, * $P\leq 0.05$. (E) Synthetic peptides ($100\mu\text{M}$) corresponding to the N-terminal extracellular domains of PAR1 (AA22-102) or PAR2(AA26-75) were incubated with rKLK10 (100 ng/mL), Thrombin (5 U/mL) or Trypsin (5 U/mL) for 30 minutes at 37°C and analyzed by Tricine SDS-PAGE. Shown is a Coomassie-stained gel representative of 3 independent studies. (F) KLK10 enzymatic activity was measured by incubating rKLK10 (60ng) with a FP-Biotin ($50 \mu\text{m}$) activity-based probe and Streptavidin-HRP Western blotting. FP-biotin was also incubated with heat-inactivated (HI) rKLK10. Binding was further assessed by a competition assay co-incubating rKLK10, FP-biotin,

and FP-alkyne ranging from 50-500 μm . (G) Quantification of F normalized to FP-Biotin+rKLK10. One-way ANOVA with Bonferroni correction. Mean $\pm$ SEM. n=3, *P $\leq$ 0.05.

FIGS. 7A-7B. KLK10 expression is decreased in human coronary arteries with atherosclerotic plaques. (A) Human coronary artery sections with or without atherosclerotic plaques were stained with KLK10 antibody and DAPI. Scale bar low mag=500 μm , scale bar; high mag=50 μm . Staining was performed for KLK10 and DAPI. Arrows indicate endothelial cells. (B) Quantification of (A) measured as fluorescent intensity. Data is from 10 different patients. Two-tailed t-test. Mean $\pm$ SEM *P $\leq$ 0.05.

FIG. 8. Flow-sensitive KLK10 inhibits inflammation and atherosclerosis indirectly through PAR1 and PAR2. KLK10 is a secreted serine protease which is upregulated by *s-flow* and downregulated by *d-flow*. KLK10 acts in a manner dependent on PAR1 and PAR2, but it does not cleave them. Without being bound by theory, KLK10 binds to an unidentified receptor, which in turn interacts with PAR1/2, leading to the inhibition of NF κ B-VCAM1-ICAM1 inflammatory signaling pathway and subsequent monocyte adhesion.

FIGS. 9A-9D. KLK10 inhibits endothelial migration and tube formation, but not apoptosis or proliferation. Human umbilical vein endothelial cells (HUVECs) were treated with rKLK10 from 0.5-100 ng/mL and (A) the scratch assay was performed to measure the rate at which endothelial cells migrated across the scratch; (B) apoptosis was assessed by TUNEL staining; (C) proliferation was assayed by ki67 immunostaining. (D) HUVECs were grown on Matrigel and treated with rKLK10 at 100 ng/mL or vehicle and tube length was measured in ImageJ. One-way ANOVA with Bonferroni correction for multiple comparisons where appropriate (A-C) or paired two-tailed t-test (D). Mean $\pm$ SEM. n=4-6 *P $\leq$ 0.05.

FIGS. 10A-10D. KLK10 reduces inflammation in endothelial cell. (A) Human aortic endothelial cells (HAECs) were transfected with KLK10 plasmid ranging from 0.01-1 $\mu\text{g/mL}$ for 24h and the THP1 monocyte adhesion assay was performed. (B) HAECs were transfected with 1 $\mu\text{g/mL}$ KLK10 plasmid for 24h and qPCR was performed to assess mRNA expression of VCAM1, ICAM1, and MCP1. (C) HAECs were treated with 0.5 to 100 ng/mL rKLK10 and monocyte adhesion assay was performed. (D) HAECs were treated with 100 ng/mL rKLK10 for 24h and qPCR was performed to assess mRNA expression of VCAM1, ICAM1, and MCP1. One-way ANOVA with Bonferroni correction for multiple comparisons (a,c) or two-way ANOVA with Bonferroni correction for multiple comparisons (b,d). Mean $\pm$ SEM. n=6. *P $\leq$ 0.05.

FIGS. 11A-11D. KLK10 plasmid and KLK10 siRNA overexpress and knockdown KLK10, respectively. (A) Human Aortic Endothelial Cells (HAECs) were transfected with 0.02-1 $\mu\text{g/mL}$ KLK10 plasmid or 1 $\mu\text{g/mL}$ GFP plasmid and KLK10 mRNA expression was measured by

qPCR. (B) HAECs were transfected with .02-1 $\mu\text{g/mL}$ KLK10 plasmid or 1 $\mu\text{g/mL}$ GFP plasmid and KLK10 secretion into the media was measured by ELISA. (C) HAECs were transfected with 500 ng/mL or 1 $\mu\text{g/mL}$ KLK10 plasmid and KLK10 protein expression was measured by western blot, using B-actin as an internal control. (D) HAECs were transfected with 25-100nM KLK10 siRNA or 100nM scrambled siRNA and KLK10 mRNA expression was measured by qPCR. n=4. Two tailed two-way ANOVA with Bonferroni correction. One-way ANOVA with Bonferroni correction for multiple comparisons where appropriate. Mean $\pm$ SEM. * $P\leq 0.05$.

FIGS. 12A-12E. rKLK10 inhibits NF κ B Activity. (A) HAECs were transfected with an NF κ B luciferase reporter plasmid and treated with TNF α (5 ng/mL) for 4h followed by rKLK10. NF κ B activity was measured as luciferase activity. (B) HAECs were treated with TNF α (5 ng/mL) for 4h followed by rKLK10 (100 ng/mL) and p65 immunostaining was performed with DAPI counterstain. Scale bar top=50 μm , scale bar bottom=10 μm . (C) Quantification of b as measure of p65 fluorescent intensity. (D) HAECs were treated with TNF α (5 ng/mL) for 4h followed by 0.1-10 ng/mL rKLK10 and expression of p-NF κ B was assessed by western blot. (E) Quantification of KLK10 signal, normalized to GAPDH and control. Data was pooled from at least three independent experiments. One-way ANOVA with Bonferroni correction for multiple comparisons where appropriate. Mean $\pm$ SEM. * $P\leq 0.05$, ** $P\leq 0.01$.

FIGS. 13A-13B. Heat-inactivation of rKLK10 prevents its anti-inflammatory effects on VCAM1 and ICAM1 expression. (A, B) HAECs were treated with TNF α (5 ng/mL) for 4h followed by 1 ng/mL, 10 ng/mL, or 10 ng/mL heat-inactivated (HI) rKLK10 overnight and mRNA expression of (A) VCAM1 and (B) ICAM1 was measured by qPCR. n=6. One-way ANOVA with Bonferroni correction for multiple comparisons. Mean $\pm$ SEM. * $P\leq 0.05$.

FIGS. 14A-14B. rKLK10 inhibits VCAM1 expression in the d-flow region of the mouse aortic arch in a dose-dependent manner. (A) Mice (male, C57/BL6) were administered 0.0006-0.6 mg/kg rKLK10 or vehicle by IV injection and inflammation was assessed by *en face* immunostaining of VCAM1 at the Lesser Curvature (LC) and the Greater Curvature (GC) of the aortic arch. Bright staining shows relative VCAM1 expression. DAPI was used for nuclear staining. Scale bar=50 μm (B) Quantification of VCAM1 staining in A normalized to the LC. n=6. Two-way ANOVA with Bonferroni correction for multiple comparisons. Mean $\pm$ SEM. * $P\leq 0.05$, ** $P\leq 0.01$.

FIGS. 15A-15C. Control studies show effective knockdown of PAR1 and PAR2 with the PAR1/2 siRNAs and the anti-inflammatory effect of rKLK10. (A, B) HAECs were transfected 50 or 100nM siPAR1, siPAR2, or control siRNA (siCtrl) and expression of PAR1 and PAR2 mRNAs were measured by qPCR. (C) HAECs were treated with rKLK10 (100 ng/mL),

TNF α (5 ng/mL), or rKLK10 and TNF α , and the THP1 monocyte adhesion to EC was performed in parallel with PAR1/2 SEAP assay shown in FIGS. 6C, D. n=4. One-way ANOVA with Bonferroni correction for multiple comparisons where appropriate. Mean $\pm$ SEM. *P $\leq$ 0.05, ***P $\leq$ 0.001.

FIG. 16. KLK10 purification from CHO cells. Wild-type, human, His-tagged KLK10 (SEQ ID NO: 3) overexpressed in CHO cells was purified by affinity chromatography. Purified KLK10 (5 ug) was resolved using SDS-PAGE under reducing (R) or non-reducing (NR) conditions and stained with Coomassie blue.

FIG. 17. Mutant KLK10 purification from CHO cells. Human, His-tagged KLK10 including Ser299Ala and Asp223Ala mutations (SEQ ID NO: 4) was overexpressed in CHO cells and then purified by affinity chromatography. Purified mutant KLK10 (5 ug) was resolved using SDS-PAGE under reducing (R) or non-reducing (NR) conditions and stained with Coomassie blue.

FIG. 18. Fc-KLK10 fusion protein purification from CHO cells. Fc-KLK10 fusion proteins (SEQ ID NO: 11 and SEQ ID NO: 13) were overexpressed separately in CHO cells and then purified by affinity chromatography. Purified fusion proteins (5 ug) were resolved using SDS-PAGE under reducing (R) or non-reducing (NR) conditions and stained with Coomassie blue.

FIG. 19. Exemplary amino acid substitutions that can be made in an Fc domain useful in any of the disclosed methods.

FIG. 20. *In vitro* monocyte adhesion assay with KLK10-Fc fusion proteins. Briefly, HAEC cells (80,000 per dish) were grown to 100% confluence in 12-well dishes. Cells were pretreated with 0.1-10 ng/ml of rKLK10 WT, rKLK10 mut, rKLK10-monomeric IgG4 Fc, rKLK10-dimeric IgG4 Fc, or a heat-inactivated version thereof, in medium with 2% FBS. Sixteen hrs later, cells were challenged with TNF α (5ng/ml) for 5 hrs. Cells were then washed and BCECF-AM ((2',7' Bis (2 Carboxyethyl) 5 (and 6))-labeled monocytes were added for 30 min at 37°C. Unbound cells were then washed off and bound monocytes were counted under a fluorescence microscope (n=4-6). Bars represent means $\pm$ SEM; *P<0.05 compared to the TNF α -treated control.

SEQUENCE LISTING

The nucleic and amino acid sequences listed in the accompanying sequence listing are shown using standard letter abbreviations for nucleotide bases, and three letter code for amino acids, as defined in 37 C.F.R. 1.822. Only one strand of each nucleic acid sequence is shown, but the complementary strand is understood as included by any reference to the displayed strand. The Sequence Listing is submitted as an ASCII text file, created on September 10, 2020, 53.4 KB, which is incorporated by reference herein. In the accompanying sequence listing:

SEQ ID NO: 1 is an amino acid sequence encoding the human KLK10 protein of GenBank Accession No. NP_001070968.1 (as available on September 8, 2020).

SEQ ID NO: 2 is an amino acid sequence encoding a human KLK10 protein.

SEQ ID NO: 3 is the amino acid sequence of **SEQ ID NO: 1**, further including a C-terminal
5 6X his tag.

SEQ ID NO: 4 is the amino acid sequence of **SEQ ID NO: 2**, further including a C-terminal
6X his tag.

SEQ ID NO: 5 is a nucleotide sequence encoding the human KLK10 gene of GenBank Accession No. NC_000019.10 (as available on September 8, 2020).

10 **SEQ ID NO: 6** is a nucleotide sequence of GenBank Accession No. NM_001077500.1 (as available on September 8, 2020) encoding the human KLK10 protein of **SEQ ID NO: 1**.

SEQ ID NO: 7 is the amino acid sequence of an exemplary Fc domain.

SEQ ID NO: 8 is the amino acid sequence of an exemplary Fc domain.

SEQ ID NO: 9 is the amino acid sequence of an exemplary Fc domain.

15 **SEQ ID NO: 10** is the amino acid sequence of an exemplary Fc domain.

SEQ ID NO: 11 is the amino acid sequence of an exemplary fusion protein.

SEQ ID NO: 12 is the amino acid sequence of an exemplary fusion protein.

SEQ ID NO: 13 is the amino acid sequence of an exemplary fusion protein.

SEQ ID NO: 14 is the amino acid sequence of an exemplary fusion protein.

20 **SEQ ID NO: 15** is a forward qPCR primer for detecting expression of human KLK10 (e.g. the KLK10 of **SEQ ID NO: 1**).

SEQ ID NO: 16 is a reverse qPCR primer for detecting expression of human KLK10 (e.g. the KLK10 of **SEQ ID NO: 1**).

SEQ ID NO: 17 is a forward qPCR primer for detecting expression of mouse KLK10.

25 **SEQ ID NO: 18** is a reverse qPCR primer for detecting expression of mouse KLK10.

SEQ ID NO: 19 is a forward qPCR primer for detecting expression of the 18S housekeeping gene.

SEQ ID NO: 20 is a reverse qPCR primer for detecting expression of the 18S housekeeping gene.

30 **SEQ ID NO: 21** is a forward qPCR primer for detecting expression of VCAM 1.

SEQ ID NO: 22 is a reverse qPCR primer for detecting expression of VCAM 1.

SEQ ID NO: 23 is a forward qPCR primer for detecting expression of ICAM1.

SEQ ID NO: 24 is a reverse qPCR primer for detecting expression of ICAM1.

SEQ ID NO: 25 is a nucleotide sequence encoding a human wild-type KLK10 with a C-

terminal His-tag.

SEQ ID NO: 26 is a nucleotide sequence including a human mutant KLK10 with a C-terminal His-tag.

SEQ ID NO: 27 is a His-tag.

5

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Atherosclerosis is an inflammatory disease that preferentially occurs in branched or curved arterial regions exposed to disturbed flow (*d-flow*), while areas of stable flow (*s-flow*) are protected from atherosclerosis (Chiu J-J & Chien S. *Physiol. Rev.* 2011, 91:327-387; Davies PF. *Physiol. Rev.* 1995, 75:519-560; Kwak BR, et al. *Eur Heart J.* 2014, 35:3013-3020, 3020a-3020d; Tarbell JM, et al. *Annu. Rev. Fluid. Mech.* 2014, 46:591-614). Endothelial cells are equipped with mechanosensors, located at the luminal and abluminal surfaces, cell-cell junctions, and cytoskeleton, which detect fluid shear stress and trigger cascades of signaling pathways and cellular responses (Kwak BR, et al. *Eur. Heart. J.* 2014, 35:3013-3020, 3020a-3020d; Tarbell JM, et al. *Annu. Rev. Fluid. Mech.* 2014, 46:591-614; Mack JJ, et al. *Nature Communications.* 2017, 8:1-19; Tzima E, et al. *Nature.* 2005, 437:426-431; Li J, et al. *The FASEB Journal.* 2015, 29:639.632; Chachisvilis M, et al. *Proc. Natl. Acad. Sci. U. S. A.* 2006, 103:15463-15468; Florian JA, et al. *Circ. Res.* 2003, 93:e136-e142; Wang L, et al. *Nature.* 2016, 540:579-582). *D-flow* induces endothelial dysfunction and atherosclerosis in large part by regulating flow-sensitive coding and non-coding genes, as wells as epigenetic modifiers (Davies PF. *Physiol. Rev.* 1995, 75:519-560; Kumar S, et al. *Arterioscler. Thromb. Vasc. Biol.* 2014, 34:2206-2216; Kumar S, et al. *Vascul. Pharmacol.* 2019, 114:76-92; Dunn J, et al. *J. Clin. Invest.* 2014, 124:3187-3199).

It is disclosed herein that Kallikrein Related Peptidase 10 (KLK10) mediates the anti-atherogenic effects of *s-flow*, while the loss of KLK10 under *d-flow* conditions leads to pro-atherogenic effects. The studies provided herein reveal that KLK10 is produced under *s-flow* in ECs but is reduced under *d-flow* conditions, and that it protects against endothelial inflammation, barrier dysfunction, and atherosclerosis.

Methods are disclosed herein for treating atherosclerosis in a subject. These methods include administering to the subject a therapeutically effective amount of a KLK10 protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein. In some embodiments, the fusion protein is a KLK10 protein fused to an Fc domain.

In some embodiments, the KLK10 protein includes an amino acid sequence at least 90% identical to SEQ ID NO: 1 or SEQ ID NO: 2. In some non-limiting examples, the KLK10 protein

comprises an amino acid sequence at least 95% identical to SEQ ID NO: 1 or SEQ ID NO: 2. In other non-limiting examples, the KLK10 protein comprises the amino acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2.

5 In additional embodiments, a fusion protein is utilized. In some non-limiting examples, the fusion protein comprises an Fc domain. The Fc domain can be monomeric or dimeric. In some embodiments, the Fc domain is a monomeric Fc domain, such as, but not limited to, an amino acid sequence at least 95% identical to SEQ ID NO: 7 or SEQ ID NO: 8. In other non-limiting examples, the monomeric Fc domain comprises the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 8. In further embodiments, the Fc domain comprises a dimerization domain, such as, but not limited to, an amino acid sequence at least 95% identical to SEQ ID NO: 9 or SEQ ID NO: 10. In some non-limiting examples, the monomeric Fc domain comprises the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10.

15 In some embodiments, the methods include administering the nucleic acid molecule encoding the KLK protein or the fusion protein to the subject. These methods can include administering to the subject a plasmid or a viral vector comprising the nucleic acid molecule encoding the KLK protein or the fusion protein. In some embodiments, the viral vector is an adeno-associated virus (AAV) vector, such as an AAV9 vector.

20 In further embodiments, the subject has atherosclerotic heart disease. In other embodiments, the subject has a stroke, peripheral artery disease or myocardial infarction. The subject can be selected for treatment. The methods can include administering a statin or niacin to the subject.

In yet other embodiments, the method inhibits monocyte adhesion to blood vessels, inhibits inflammation in blood vessels, and/or protects the endothelial permeability barrier in blood vessels in the subject.

25 In some embodiments, the method includes administering the KLK10 protein, the fusion protein, or the nucleic molecule, locally to a vessel of the subject. The KLK10 protein, the fusion protein, or the nucleic molecule, can be administered in a stent.

30 In some embodiments, methods are disclosed for decreasing arterial endothelial inflammation in a subject. These methods include selecting a subject with atherosclerosis, and administering to the subject a therapeutically effective amount of a KLK10 protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, thereby decreasing arterial endothelial inflammation in the subject. The KLK10 protein can include an amino acid sequence at least 90% identical to SEQ ID NO: 1 or SEQ ID NO: 2, such as an amino acid sequence at least 95% identical to SEQ ID NO: 1 or SEQ ID NO: 2, or an amino

acid sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In some non-limiting examples, the fusion protein comprises an Fc domain. The Fc domain can be a monomeric Fc domain, such as an amino acid sequence at least 95% identical to SEQ ID NO: 7 or SEQ ID NO: 8, or the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 8. In other non-limiting examples, the Fc domain comprises a dimerization domain, such as an amino acid sequence at least 95% identical to SEQ ID NO: 9 or SEQ ID NO: 10, or the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10. In other embodiments, these methods include administering the nucleic acid molecule encoding the KLK10 protein or the fusion protein to the subject. The subject can have atherosclerosis. In yet other embodiments, the subject has atherosclerotic heart disease. In more embodiments, the method includes administering to the subject a therapeutically effective amount of a statin or niacin.

In more embodiments, the subject is administered a plasmid or a viral vector comprising the nucleic acid molecule encoding the KLK protein or the fusion protein. The viral vector can be an adeno-associated virus (AAV) vector, such as, but not limited to, an AAV9 vector.

In yet other embodiments, the subject is administered the KLK10 protein, the fusion protein, or the nucleic molecule, locally to the vessel of the subject. The KLK10 protein, the fusion protein, or the nucleic molecule, can be provided in a stent.

In further embodiments, a composition is provided that includes a KLK10 protein that has an amino acid sequence at least 90% identical to SEQ ID NO: 1 or SEQ ID NO: 2, a fusion protein including the KLK10 protein, or a nucleic acid molecule, such as an expression vector, encoding the KLK protein or the fusion protein. This composition is of use in any of the methods disclosed herein.

In any of the disclosed embodiments, the KLK10 protein may optionally include a His-tag.

Summary of Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of many common terms in molecular biology may be found in Krebs *et al.* (eds.), *Lewin's genes XII*, published by Jones & Bartlett Learning, 2017. As used herein, the singular forms "a," "an," and "the," refer to both the singular as well as plural, unless the context clearly indicates otherwise. For example, the term "an antigen" includes singular or plural antigens and can be considered equivalent to the phrase "at least one antigen." As used herein, the term "comprises" means "includes." It is further to be understood that any and all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or proteins are approximate, and are provided for descriptive purposes, unless otherwise indicated. Although

many methods and materials similar or equivalent to those described herein can be used, particular suitable methods and materials are described herein. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. To facilitate review of the various embodiments, the following explanations of terms are provided:

Adeno-associated Virus (AAV): AAV is a small virus that infects humans and some other primate species. AAV is not currently known to cause disease and consequently the virus causes a very mild immune response. AAV can infect both dividing and non-dividing cells and mainly exists as episomal forms in the host cell. The AAV genome is built of single-stranded deoxyribonucleic acid (ssDNA), either positive- or negative-sensed, which is about 4.7 kilobases (kb) long. The genome comprises inverted terminal repeats (ITRs) at both ends of the DNA strand, and two open reading frames (ORFs): *rep* and *cap*. *Rep* is composed of four overlapping genes encoding Rep proteins required for the AAV life cycle, and *Cap* contains overlapping nucleotide sequences of capsid proteins: VP1, VP2 and VP3, which interact together to form a capsid of an icosahedral symmetry. For gene therapy, ITRs seem to be the only sequences required *in cis* next to the therapeutic gene: structural (*cap*) and packaging (*rep*) genes can be delivered *in trans*.

About: Unless context indicated otherwise, “about” refers to plus or minus 5% of a reference value. For example, “about” 100 refers to 95 to 105.

Administration: The introduction of a composition into a subject by a chosen route. Administration can be local or systemic. For example, if the chosen route is intravenous, the composition is administered by introducing the composition into a vein of the subject. Exemplary routes of administration include, but are not limited to, oral, injection (such as subcutaneous, intramuscular, intradermal, intraperitoneal, and intravenous), sublingual, rectal, transdermal (for example, topical), intranasal, vaginal, and inhalation routes.

Alter: A change in an effective amount of a substance of interest, such as a polynucleotide or polypeptide. The amount of the substance can be changed by a difference in the amount of the substance produced, by a difference in the amount of the substance that has a desired function, or by a difference in the activation of the substance. The change can be an increase or a decrease. The alteration can be *in vivo* or *in vitro*.

In several embodiments, altering an amount of a polypeptide or polynucleotide is at least about a 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% increase or decrease in the effective amount (level) of a substance. In specific example, an increase of a polypeptide or polynucleotide is at least about a 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% increase in a polypeptide or polynucleotide as compared to a control, a statistical normal, or a

standard value chosen for specific study. In another specific example, a decrease of a polypeptide or polynucleotide, such as following the initiation of a therapeutic protocol, is at least about a 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% decrease in a polypeptide or polynucleotide as compared to a control, a statistical normal, or a standard value chosen for specific study.

Atherosclerosis: An inflammatory disease that preferentially occurs in branched or curved arterial regions exposed to disturbed blood flow (*d-flow*), while areas of stable blood flow (*s-flow*) are generally protected from atherosclerosis. Atherosclerosis includes the progressive narrowing and hardening of a blood vessel over time. Atherosclerosis is a common form of arteriosclerosis in which deposits of yellowish plaques (atheromas) containing cholesterol, lipid material and lipophages are formed within the intima and inner media of large and medium-sized arteries. Treatment of atherosclerosis includes reversing or slowing the progression of atherosclerosis, for example as measured by the presence of atherosclerotic lesions and/or functional signs of the disease, such as improvement in cardiovascular function as measured by signs (such as peripheral capillary refill), symptoms (such as chest pain and intermittent claudication), or laboratory evidence (such as that obtained by EKG, angiography, or other imaging techniques).

Cardiovascular: Pertaining to the heart and/or blood vessels.

Cardiovascular disease (CVD): Disorders of the heart and blood vessels, such as atherosclerosis (ASCVD), coronary heart disease, cerebrovascular disease, and peripheral vascular disease. Cardiovascular diseases also include, for example, myocardial infarction, stroke, angina pectoris, transient ischemic attacks, and congestive heart failure. Atherosclerosis usually results from the accumulation of fatty material, inflammatory cells, extracellular matrices and plaque. Clinical symptoms and signs indicating the presence of CVD may include one or more of the following: chest pain and other forms of angina, shortness of breath, sweateness, Q waves or inverted T waves on an EKG, a high calcium score by CT scan, at least one stenotic lesion on coronary angiography, and heart attack. Subclinical ASCVD can be identified by imaging tests (such as CT measures of coronary calcification, or MRI measures of coronary or aortic plaque, and/or ultrasound evidence of carotid plaque or thickening).

Cholesterol absorption inhibitor: A class of cholesterol lowering drugs that block absorption of cholesterol at the brush border of the intestine without affecting absorption of triglycerides or fat-soluble vitamins. These drugs are not systemically absorbed and can lower cholesterol on their own (*i.e.* without the use of additional drugs). An exemplary cholesterol absorption inhibitor is ezetimibe (Ezetrol).

Cholesterol lowering agent: An agent that lowers the level of cholesterol in a subject, such as a pharmaceutical, vitamin, or small molecule. One of skill in the art can readily identify assays, such as blood screening, to determine the effect of cholesterol. Agents include, but are not limited to, niacin, the statins (*e.g.*, ZOCOR™, LIPITOR™, PRAVACOL™, LESCOR™, MEVACOR™), bile acid binding resins (*e.g.*, QUESTRAN™), and fibrates (*e.g.* LOPID™, LIPIDIL MICRO™).

Conservative variants: “Conservative” amino acid substitutions are those substitutions that do not substantially affect or decrease a function of a protein, such as the effect of KLK0 on atherosclerosis. For example, a KLK10 protein can include up to 1, 2, 3, 4, 5, 6, 7, 8, 9, or up to 10 conservative substitutions compared to a reference KLK10 sequence and can inhibit arterial endothelial inflammation. The term conservative variation also includes the use of a substituted amino acid in place of an unsubstituted parent amino acid.

Individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (for instance less than 5%, in some embodiments less than 1%) in an encoded sequence are conservative variations where the alterations result in the substitution of an amino acid with a chemically similar amino acid.

The following six groups are examples of amino acids that are considered to be conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

Non-conservative substitutions are those that reduce an activity or function of KLK10, such as reducing its anti-inflammatory activity. For instance, if an amino acid residue is essential for a function of the protein, even an otherwise conservative substitution may disrupt that activity. Thus, a conservative substitution does not alter the basic function of a protein of interest.

Control: A “control” refers to a sample or standard used for comparison with an experimental sample. In some embodiments, the control is a sample obtained from a healthy patient or a non-diseased tissue sample obtained from a patient diagnosed with the disorder of interest, such as MI or ASCVD. In some embodiments, the control is a historical control or standard reference value or range of values (such as a previously tested control sample, such as a group of patients with the disorder, or group of samples that represent baseline or normal values,

such as the level of specific genes in non-diseased tissue).

Disturbed Flow (d-flow): Blood flow in geometrically irregular arterial regions such as curvatures, branches, and bifurcations that are characterized by overall low shear stress combined with high retrograde flow and oscillatory shear stress. In contrast, **stable flow (s-flow)** is blood flow that occurs in long straight regions of the blood vessels. Sustained (s-flow) with high shear stress upregulates expressions of endothelial genes and proteins that are protective against atherosclerosis, whereas disturbed flow with associated reciprocating, low shear stress generally upregulates the endothelial genes and proteins that promote atherogenesis.

Expression: Transcription or translation of a nucleic acid sequence. For example, an encoding nucleic acid sequence (such as a gene) can be expressed when its DNA is transcribed into RNA or an RNA fragment, which in some examples is processed to become mRNA. An encoding nucleic acid sequence (such as a gene) may also be expressed when its mRNA is translated into an amino acid sequence, such as a protein or a protein fragment. In a particular example, a heterologous gene is expressed when it is transcribed into an RNA. In another example, a heterologous gene is expressed when its RNA is translated into an amino acid sequence. Regulation of expression can include controls on transcription, translation, RNA transport and processing, degradation of intermediary molecules such as mRNA, or through activation, inactivation, compartmentalization or degradation of specific protein molecules after they are produced.

Expression Control Sequences: Nucleic acid sequences that regulate the expression of a heterologous nucleic acid sequence to which it is operatively linked. Expression control sequences are operatively linked to a nucleic acid sequence when the expression control sequences control and regulate the transcription and, as appropriate, translation of the nucleic acid sequence. Thus, expression control sequences can include appropriate promoters, enhancers, transcriptional terminators, a start codon (ATG) in front of a protein-encoding gene, splice signals for introns, maintenance of the correct reading frame of that gene to permit proper translation of mRNA, and stop codons. The term "control sequences" is intended to include, at a minimum, components whose presence can influence expression, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences. Expression control sequences can include a promoter.

Expression vector: A vector comprising a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient cis- acting elements for expression; other elements for expression can be supplied by the host cell or in an in vitro expression system. Non-limiting

examples of expression vectors include cosmids, plasmids (*e.g.*, naked or contained in liposomes) and viruses (*e.g.*, lentiviruses, retroviruses, adenoviruses, and adeno-associated viruses) that incorporate the recombinant polynucleotide.

A polynucleotide encoding KLK10 or a fusion protein encoding KLK10 can be inserted into an expression vector that contains a promoter sequence which facilitates the efficient transcription of the inserted genetic sequence of the host. The expression vector typically contains an origin of replication, a promoter, as well as specific nucleic acid sequences that allow phenotypic selection of the transformed cells.

Fragment crystallizable (Fc) region: The constant region of an antibody excluding the first heavy chain constant domain. Fc region generally refers to the last two heavy chain constant domains of IgA, IgD, and IgG, and the last three heavy chain constant domains of IgE and IgM. An Fc region may also include part or all of the flexible hinge N-terminal to these domains. For IgA and IgM, an Fc region may or may not include the tailpiece, and may or may not be bound by the J chain. For IgG, the Fc region is typically understood to include immunoglobulin domains C γ 2 and C γ 3 and optionally the lower part of the hinge between C γ 1 and C γ 2. Although the boundaries of the Fc region may vary, the human IgG heavy chain Fc region is usually defined to include residues following C226 or P230 to the Fc carboxyl-terminus, wherein the numbering is according to Kabat. For IgA, the Fc region includes immunoglobulin domains C α 2 and C α 3 and optionally the lower part of the hinge between C α 1 and C α 2. The domains of any Fc can be identified using the International ImMunoGene Tics information system (IMGT), through the internet, at imgt.cines.fr/. The binding sites for C1q and Fc γ R are located in the CH2 domain of IgG. The glutamic acid, lysine, and lysine residues at positions 318, 320, and 322 (IMGT numbering), respectively, are a binding motif for C1q. In addition, amino acid residues at positions 234–238 play a role in the high-affinity interaction of murine IgG2a with Fc γ RI.

Framingham Risk Score: A risk factor score that is used for predicting future risk of coronary artery disease in individuals free of disease, based on the measurement of Framingham risk factors which include age, gender, systolic blood pressure (and use of antihypertensive treatment), cigarette smoking, diabetes, as well as total cholesterol (or low density lipoprotein cholesterol (LDL cholesterol) and high density lipoprotein cholesterol (HDL cholesterol) levels (Wilson *et al.*, *Circulation* 1998; 97: 1837- 47).

Fusion Protein: A protein comprising at least two heterologous domains that are not present together in nature. Each domain of a fusion protein is encoded by a separate nucleic acid molecule. In some embodiments, at least two of such nucleic acid molecules are joined so that they are transcribed and translated as a single unit, producing a single fusion protein. The separate

nucleic acid molecules may be joined end-to-end or may be joined using a linker sequence, such as a flexible linker comprising multiple glycine residues or a rigid linker comprising proline residues. A linker may be cleavable, such as *in vivo*, such as to allow release of one or more fused domains under certain conditions, such as a change in pH or interaction with a specific biological molecule.

5 Fusion proteins can also be designed to allow for post-translational conjugation of the at least two domains of interest, in contrast to fusion of nucleic acid molecules prior to translation. Generally, a fusion protein is engineered to modify the properties of one or more of the domains of the fusion protein. A fusion protein can comprise, for example, an Fc domain and a therapeutic molecule, such as KLK10 or a variant thereof. Fusions of an Fc domain and a therapeutic molecule, such as a
10 KLK10 protein, can increase the half-life of the therapeutic molecule.

Heterologous: A heterologous sequence is a sequence that is not normally (in the wild-type sequence) found adjacent to a second sequence. In one embodiment, the sequence is from a different genetic source, such as a virus or organism, than the second sequence. In another embodiment, the heterologous sequence is a recombinant sequence that is not normally next to the
15 wild-type sequence, such as KLK10 and an Fc domain.

His-tag: A “His-tag” is an amino acid motif that typically consists of at least six histidine (His) residues and no more than ten His residues, often at the N- or C-terminus of the protein. A His-tag may also be referred to as a polyhistidine tag, a hexa-histidine-tag, a 6X His-tag, or a His6 tag. An exemplary His-tag sequence is provided in SEQ ID NO: 27 (VDHHHHHH). His-tags are
20 often used for affinity purification of His-tagged recombinant proteins, such as a His-tagged KLK10 protein, produced in cell culture systems.

Host cell: Cells in which a vector can be propagated and its DNA expressed. The cell may be prokaryotic or eukaryotic. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations
25 that occur during replication. However, such progeny are included when the term “host cell” is used.

Inhibiting or treating a disease: Inhibiting the full development of a disease or condition, for example, in a subject who is at risk for a disease such as stroke, myocardial infarction, or peripheral vascular disease. “**Treatment**” refers to a therapeutic intervention that ameliorates a
30 sign or symptom of a disease or pathological condition after it has begun to develop, such as a sign or symptom of atherosclerotic heart disease. Treatment can also induce remission or cure of a condition, such as atherosclerotic heart disease. The term “**ameliorating**,” with reference to a disease or pathological condition, refers to any observable beneficial effect of the treatment. The beneficial effect can be evidenced, for example, by a delayed onset of clinical symptoms of the

disease in a susceptible subject, a reduction in severity of some or all clinical symptoms of the disease, a slower progression of the disease, an improvement in the overall health or well-being of the subject, or by other parameters well known in the art that are specific to the particular disease. For example, ameliorating the signs, symptoms, or complications of atherosclerosis may include decreasing the size or number of atherosclerotic plaques in the subject, decreasing the cholesterol content of an atherosclerotic plaque in the subject, and/or reducing the subject's total plasma cholesterol, free cholesterol, cholesterol ester, very low-density lipoprotein cholesterol (VLDL-C), low density lipoprotein cholesterol (LDL-C), and/or phospholipids, such as compared to the subject prior to the treatment or as compared to an untreated subject. A "**prophylactic**" treatment is a treatment administered to a subject who does not exhibit signs of a disease or exhibits only early signs for the purpose of decreasing the risk of developing pathology, such as atherosclerosis. "**Inhibiting**" of a disease does not require a total absence of the disease, such as atherosclerotic heart disease. For example, a decrease of at least 50% can be sufficient.

Isolated: A biological component (such as a nucleic acid, peptide, protein or protein complex, for example an antibody) that has been substantially separated, produced apart from, or purified away from other biological components in the cell of the organism in which the component naturally occurs, that is, other chromosomal and extra-chromosomal DNA and RNA, and proteins. Thus, isolated nucleic acids, peptides and proteins include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids, peptides and proteins prepared by recombinant expression in a host cell, as well as, chemically synthesized nucleic acids. An isolated nucleic acid, peptide or protein, for example an antibody, can be at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% pure.

Kallikrein-Related Peptidase 10 (KLK10): An epithelial cell-specific 1 (NES1) protein that is a member of the kallikrein-related peptidase 'KLK' family of 15 secreted serine proteases, which are found as a gene cluster on human chromosome (19q13.4). The KLKs are distinct from plasma kallikrein, which is encoded on a separate chromosome (4q35). Despite the chromosomal clustering of the KLKs, each enzyme has a unique tissue expression pattern with different cellular functions. Typically, the KLKs are produced as inactive full-length pre-pro-proteins, which are then secreted and activated by a complex process to yield active extracellular enzyme. Like plasma kallikrein, certain KLKs can act on kininogen to generate kinins that can affect vascular endothelial function.

KLK10 is overexpressed in ovarian, pancreatic, and uterine cancer. Since KLK10 level can be easily measured in blood, studies have shown abnormal serum levels of KLK10 in patients with

breast, prostate, or ovarian cancer, suggesting its tissue-specific pathophysiological roles. An exemplary amino acid sequence encoding a KLK10 pre-pro-protein is provided in GenBank Accession No. NP_001070968.1 incorporated herein by reference, and as UniProt No. O43240, incorporated herein by reference, and an exemplary nucleotide sequence encoding the human
5 KLK10 gene is provided in GenBank Accession No. NC_000019.10, incorporated herein by reference, all as available on September 8, 2020.

Myocardial Infarction (MI): An event that occurs when blood stops flowing properly to part of the heart and the heart muscle is injured due to inadequate oxygen delivery. Acute myocardial infarction refers to two subtypes of acute coronary syndrome, namely non-ST-elevated
10 myocardial infarction and ST-elevated myocardial infarction, which are most frequently (but not always) a manifestation of coronary artery disease. The most common triggering event is the disruption of an atherosclerotic plaque in an epicardial coronary artery, which leads to a clotting cascade, sometimes resulting in total occlusion of the artery. If impaired blood flow to the heart lasts long enough, it triggers a process called the ischemic cascade; the heart cells in the territory of
15 the occluded coronary artery die, chiefly through necrosis. A collagen scar forms in the heart in place of the damaged cells.

Niacin: A B-vitamin that is used as a medication for patients with elevated levels of triglycerides and cholesterol. A long-acting preparation of niacin is available as NIASPAN®.

Nucleic acid (molecule or sequence): A deoxyribonucleotide or ribonucleotide polymer or
20 combination thereof including without limitation, cDNA, mRNA, genomic DNA, and synthetic (such as chemically synthesized) DNA or RNA. The nucleic acid can be double stranded (ds) or single stranded (ss). Where single stranded, the nucleic acid can be the sense strand or the antisense strand. Nucleic acids can include natural nucleotides (such as A, T/U, C, and G), and can include analogs of natural nucleotides, such as labeled nucleotides.

25 “cDNA” refers to a DNA that is complementary or identical to an mRNA, in either single stranded or double stranded form.

“Encoding” refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of
30 nucleotides (i.e., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and non-coding strand, used as the template for transcription, of a

gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a “nucleotide sequence encoding an amino acid sequence” includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

5 **Operably linked:** A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter, such as the CMV promoter, is operably linked to a coding sequence if the promoter affects the transcription or expression of the coding sequence. Generally, operably linked DNA sequences are contiguous and, where necessary to join
10 two protein-coding regions, in the same reading frame.

Peripheral Vascular Disease (PVD): A condition in which the arteries that carry blood to the arms or legs become narrowed or occluded. This interferes with the normal flow of blood, sometimes causing pain but often causing no readily detectable symptoms at all.

 The most common cause of PVD is atherosclerosis, a gradual process in which cholesterol
15 and scar tissue build up, forming plaques that occlude the blood vessels. In some cases, PVD may be caused by blood clots that lodge in the arteries and restrict blood flow. PVD affects about one in 20 people over the age of 50, or 8 million people in the United States. More than half the people with PVD experience leg pain, numbness or other symptoms, but many people dismiss these signs as “a normal part of aging” and do not seek medical help. The most common symptom of PVD is
20 painful cramping in the leg or hip, particularly when walking. This symptom, also known as “claudication,” occurs when there is not enough blood flowing to the leg muscles during exercise, such that ischemia occurs. The pain typically goes away when the muscles are rested.

 Other symptoms may include numbness, tingling or weakness in the leg. In severe cases, people with PVD may experience a burning or aching pain in an extremity such as the foot or toes
25 while resting, or may develop a sore on the leg or foot that does not heal. People with PVD also may experience a cooling or color change in the skin of the legs or feet, or loss of hair on the legs. In extreme cases, untreated PVD can lead to gangrene, a serious condition that may require amputation of a leg, foot or toes. People with PVD are also at higher risk for heart disease and stroke.

30 **Pharmaceutically acceptable carriers:** The pharmaceutically acceptable carriers of use are conventional. *Remington: The Science and Practice of Pharmacy*, 22nd ed., London, UK: Pharmaceutical Press, 2013, describes compositions and formulations suitable for pharmaceutical delivery of the disclosed agents.

 In general, the nature of the carrier will depend on the particular mode of administration

being employed. For instance, parenteral formulations usually include injectable fluids that include pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (*e.g.*, powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch, or magnesium stearate. In addition to biologically neutral carriers, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary substances, such as wetting or emulsifying agents, added preservatives (such as non-natural preservatives), and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate. In particular examples, the pharmaceutically acceptable carrier is sterile and suitable for parenteral administration to a subject for example, by injection. In some embodiments, the active agent and pharmaceutically acceptable carrier are provided in a unit dosage form such as a pill or in a selected quantity in a vial. Unit dosage forms can include one dosage or multiple dosages (for example, in a vial from which metered dosages of the agents can selectively be dispensed).

Pharmaceutical agent: A chemical compound or composition, such as including a nucleic acid molecule, capable of inducing a desired therapeutic or prophylactic effect when properly administered to a subject or a cell. "Incubating" includes a sufficient amount of time for a drug to interact with a cell. "Contacting" includes incubating a drug in solid or in liquid form with a cell.

Polypeptide: A polymer in which the monomers are amino acid residues that are joined together through amide bonds. When the amino acids are alpha-amino acids, either the L-optical isomer or the D-optical isomer can be used, the L-isomers being preferred. The terms "polypeptide" or "protein" as used herein are intended to encompass any amino acid sequence and include modified sequences such as glycoproteins. A polypeptide includes both naturally occurring proteins, as well as those that are recombinantly or synthetically produced. A polypeptide has an amino terminal (N-terminal) end and a carboxy-terminal end. In some embodiments, the polypeptide is a disclosed antibody or a fragment thereof.

Purified: The term purified does not require absolute purity; rather, it is intended as a relative term. Thus, for example, a purified peptide preparation is one in which the peptide or protein (such as an antibody) is more enriched than the peptide or protein is in its natural environment within a cell. In one embodiment, a preparation is purified such that the protein or peptide represents at least 50% of the total peptide or protein content of the preparation.

Promoter: A promoter is an array of nucleic acid control sequences which direct transcription of a nucleic acid. A promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A

promoter also optionally includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription.

A promoter can be a constitutively active promoter (i.e., a promoter that is constitutively in an active/"ON" state), an inducible promoter (i.e., a promoter whose state, active/"ON" or inactive/"OFF", is controlled by an external stimulus, e.g., the presence of a particular temperature, compound, or protein.), a spatially restricted promoter (e.g., tissue specific promoter, cell type specific promoter, etc.), or it may be a temporally restricted promoter (i.e., the promoter is in the "ON" state or "OFF" state during specific stages of embryonic development or during specific stages of a biological process).

Recombinant: A recombinant nucleic acid is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. This artificial combination can be accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques. A recombinant protein is one that has a sequence that is not naturally occurring or has a sequence that is made by an artificial combination of two otherwise separated segments of sequence. In several embodiments, a recombinant protein is encoded by a heterologous (for example, recombinant) nucleic acid that has been introduced into a host cell, such as a bacterial or eukaryotic cell. The nucleic acid can be introduced, for example, on an expression vector having signals capable of expressing the protein encoded by the introduced nucleic acid or the nucleic acid can be integrated into the host cell chromosome.

Sequence identity: The identity between two or more nucleic acid sequences, or two or more amino acid sequences, is expressed in terms of the identity between the sequences. Sequence identity can be measured in terms of percentage identity; the higher the percentage, the more identical the sequences. Homologs and variants of a V_L or a V_H of an antibody that specifically binds a target antigen are typically characterized by possession of at least about 75% sequence identity, for example at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity counted over the full-length alignment with the amino acid sequence of interest.

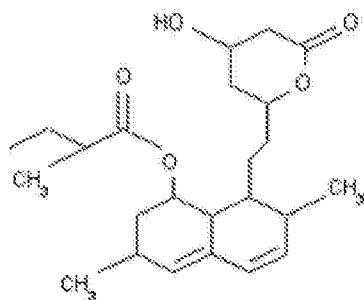
Any suitable method may be used to align sequences for comparison. Non-limiting examples of programs and alignment algorithms are described in: Smith and Waterman, *Adv. Appl. Math.* 2(4):482-489, 1981; Needleman and Wunsch, *J. Mol. Biol.* 48(3):443-453, 1970; Pearson and Lipman, *Proc. Natl. Acad. Sci. U.S.A.* 85(8):2444-2448, 1988; Higgins and Sharp, *Gene*, 73(1):237-244, 1988; Higgins and Sharp, *Bioinformatics*, 5(2):151-3, 1989; Corpet, *Nucleic Acids Res.* 16(22):10881-10890, 1988; Huang *et al. Bioinformatics*, 8(2):155-165, 1992; and Pearson,

Methods Mol. Biol. 24:307-331, 1994. Altschul *et al.*, *J. Mol. Biol.* 215(3):403-410, 1990, presents a detailed consideration of sequence alignment methods and homology calculations. The NCBI Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, *J. Mol. Biol.* 215(3):403-410, 1990) is available from several sources, including the National Center for Biological Information and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn, and tblastx. Blastn is used to compare nucleic acid sequences, while blastp is used to compare amino acid sequences. Additional information can be found at the NCBI web site.

Generally, once two sequences are aligned, the number of matches is determined by counting the number of positions where an identical nucleotide or amino acid residue is present in both sequences. The percent sequence identity between the two sequences is determined by dividing the number of matches either by the length of the sequence set forth in the identified sequence, or by an articulated length (such as 100 consecutive nucleotides or amino acid residues from a sequence set forth in an identified sequence), followed by multiplying the resulting value by 100.

Statin: Any of a class of lipid-lowering drugs that reduce serum cholesterol levels by inhibiting a key enzyme involved in the biosynthesis of cholesterol. Example statins include atorvastatin (LIPITOR®), fluvastatin (LESCOL®), lovastatin (MEVACOR®, ALTOCOR®, not marketed in the UK), pravastatin (PRAVACHOL®, SELEKTINE®, LIPOSTAT®), rosuvastatin (CRESTOR®), simvastatin (ZOCOR®). There are two groups of statins: (1) Fermentation-derived: lovastatin, simvastatin and pravastatin, and (2) Synthetic statins: fluvastatin, atorvastatin, cerivastatin and rosuvastatin. Generally, statins act by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, an enzyme of the HMG-CoA reductase pathway, the body's metabolic pathway for the synthesis of cholesterol.

The structure of one exemplary statin, lovastatin, is shown below.



Stroke: A stroke occurs when a portion of the brain has insufficient blood flow, leading to cell death. Stroke can be caused by thrombus formation in the carotid arteries. Ischemic stroke occurs when a blood vessel in the brain becomes at least partially blocked, preventing a full supply of blood from passing the blockage to reach other areas of the brain, and leading to dysfunction of the brain tissue in that area. Ischemic stroke may occur, for example, due to thrombosis

(obstruction of a blood vessel by a blood clot forming locally), embolism (obstruction due to an embolus from elsewhere in the body), systemic hypoperfusion (general decrease in blood supply, e.g., in shock), and/or cerebral venous sinus thrombosis.

5 Hemorrhagic stroke occurs when a blood vessel in the brain ruptures or leaks. This can prevent other areas of the brain from receiving adequate blood flow, and can create pressure and other injuries in the area of the rupture or leak. Hemorrhagic strokes generally include intracerebral hemorrhage or subarachnoid hemorrhage. Intracerebral hemorrhage is bleeding within the brain itself (wherein an artery in the brain bursts, flooding the surrounding tissue with blood) due to either intraparenchymal hemorrhage (bleeding within the brain tissue) or intraventricular
10 hemorrhage (bleeding within the brain's ventricular system). Subarachnoid hemorrhage is bleeding that occurs outside of the brain tissue but still within the skull, and between the arachnoid mater and pia mater.

Subject: Living multi-cellular vertebrate organisms, a category that includes human and non-human mammals. In an example, a subject is a human. A subject can be in need of treatment,
15 such as a subject with atherosclerosis.

Therapeutically effective amount: An amount of a pharmaceutical preparation that alone, or together with a pharmaceutically acceptable carrier or one or more additional therapeutic agents, induces the desired response. A therapeutic agent, such as an anticoagulant, or a statin, is administered in therapeutically effective amounts.

20 Effective amounts a therapeutic agent can be determined in many different ways, such as assaying for a reduction in atherosclerotic disease or improvement of physiological condition of a subject having vascular disease. Effective amounts also can be determined through various *in vitro*, *in vivo* or *in situ* assays.

Therapeutic agents can be administered in a single dose, or in several doses, for example
25 daily, during a course of treatment. However, the effective amount of can be dependent on the source applied, the subject being treated, the severity and type of the condition being treated, and the manner of administration.

In one example, it is an amount sufficient to partially or completely alleviate symptoms of atherosclerosis and/or vascular disease within a subject. Treatment can involve only slowing the
30 progression of the atherosclerosis and/or vascular disease temporarily, but can also include halting or reversing the progression of the atherosclerosis and/ vascular disease permanently. For example, a pharmaceutical preparation can decrease one or more symptoms of vascular disease, for example decrease a symptom by at least 20%, at least 50%, at least 70%, at least 90%, at least 98%, or even at least 100%, as compared to an amount in the absence of the pharmaceutical preparation.

Vector: A nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in the host cell, such as an origin of replication. A vector may also include one or more therapeutic genes and/or selectable marker genes and other genetic elements known in the art. A vector can transduce, transform or infect a cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell. A vector optionally includes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like.

Virus: Microscopic infectious organism that reproduces inside living cells. A virus consists essentially of a core of a single nucleic acid surrounded by a protein coat and has the ability to replicate only inside a living cell. "Viral replication" is the production of additional virus by the occurrence of at least one viral life cycle. Viral vectors are known in the art, and include, for example, adenovirus, AAV, lentivirus and herpes virus.

Unless explained otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this disclosure belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, suitable methods and materials are described below. The materials, methods, and examples are illustrative only and not intended to be limiting.

KLK10

The disclosed methods use a therapeutically effective amount of a Kallikrein Related Peptidase 10 (KLK10) protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, to treat atherosclerosis in the subject. KLK10 is an epithelial cell-specific 1 (NES1) protein that is a member of the kallikrein-related peptidase 'KLK' family of 15 secreted serine proteases, which *in vivo* in humans are found as a gene cluster on human chromosome (19q13.4). Despite the chromosomal clustering of the KLKs, each enzyme has a unique tissue expression pattern with different cellular functions. Typically, the KLKs are produced as inactive full-length pre-pro-proteins, which are secreted and activated by a complex process to yield active extracellular enzyme. Certain KLKs can act on kininogen to generate kinins that can affect vascular endothelial function.

Human KLK10 is classified as a serine protease. KLK10 is synthesized as an inactive pre-pro-protein that is proteolytically processed into a secreted inactive pro-protein. Subsequently, the KLK10 pro-protein is activated to the mature peptidase by proteolytic removal of the N-terminal

propeptide. The proteolytic activities of proteases are known to be regulated by this secondary cleavage process. KLK10 is produced as a pre-pro-protein. A KLK10 pre-pro-protein, a KLK10 pro-protein, or the mature KLK10 protein can be used in the disclosed methods. As used herein, a “KLK10 protein” refers to any of these forms. The KLK10 protein can be from any species, including, but not limited to, a human KLK10 protein.

In any embodiment, a protein sequence for purification, such as a histidine tag, can be added to the KLK10 protein. In specific non-limiting examples, a 6X histidine tag can be added at the C-terminus or the N-terminus of the KLK10 protein. This protein sequence can be added at the C-terminus or the N-terminus of the KLK-protein. An exemplary protein sequence for purification is shown in **SEQ ID NO: 27**. Thus, in some embodiments, the KLK10 protein includes a histidine tag.

In some embodiments, the KLK10 protein comprises one or more amino acid modifications (i.e., substitutions, deletions, and/or additions), such as, for example, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions. Thus, of use in the disclosed methods are KLK10 pre-pro-proteins comprising at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions, KLK10 pro-proteins comprising at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions, and mature KLK10 comprising at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions. In other embodiments, the KLK10 protein is about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identical to a wild-type pre-pro-protein, pro-protein or mature KLK10 protein wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, and/or atherosclerosis.

In some non-limiting examples, substitutions can be made in the conserved catalytic triad region and/or in the conserved binding pocket region. In other non-limiting examples, substitutions can be made in domain(s) required for protease activity. Suitable mutations can be made for example, at residues 223 and 229. In some non-limiting examples, the aspartic acid at residue 223 can be replaced with alanine, and/or the serine at residue 229 can be substituted with alanine, which are substitutions in the substrate binding pocket region mutations of the KLK10 protein. These mutations in residues 223 and 229 resulted in loss of proteolytic activity in the mature protein; however, there is no change in the biological activity with regard to efficacy in the claimed methods.

In some embodiments, a pre-pro-protein KLK10 has the following the amino acid sequence:

MRAPHLHLSAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDPEAYGSPCARGSQP
WQVSLFNGLSFHCAGVLVDQSWVLTAHCGNKPLWARVGDDHLLLLQGEQLRRTTRSV

VHPKYHQSGPILPRRTDEHDLMLLKLARPVVLGPRVRALQLPYRCAQPGDQCQVAGWG
 TTAARRVKYNKGLTCSSITILSPKECEVFYPGVVTNNMICAGLDRGQDPCQSDSGGPLVCD
 ETLQGILSWGVPYPCGSAQHPAVYTQICKYMSWINKVIRSN (**SEQ ID NO: 1**, see
 GENBANK® Accession No. NP_001070968.1, incorporated herein by reference as of September
 8, 2020), wherein the underlined sequence (amino acids 1-33) is the KLK10 pre-peptide, the bolded
 sequence (amino acids 34-42) is the KLK10 pro-peptide, and the remainder of the sequence (amino
 acids 43-276) represents the mature KLK10 protein. Any of these forms can be used in the
 presently disclosed methods. For any amino acid sequence disclosed herein that bears an N-
 terminal methionine, this residue is optional and may be removed.

In some embodiments, a KLK10 protein is:

MRAPHLHLSAASGARALAKLLPLLMAQLWAAEA**ALLPQND**TRLDPEAYGSPCARGSQP
 WQVSLFNGLSFHCAGVLVDQSWVLTAAHCGNKPLWARVGDDHLLLQGEQLRRRTTRSV
 VHPKYHQSGPILPRRTDEHDLMLLKLARPVVLGPRVRALQLPYRCAQPGDQCQVAGWG
 TTAARRVKYNKGLTCSSITILSPKECEVFYPGVVTNNMICAGLDRGQAPCQSDAGGPLVCD
 ETLQGILSWGVPYPCGSAQHPAVYTQICKYMSWINKVIRSN (**SEQ ID NO: 2**),

wherein the underlined sequence (amino acids 1-33) represents the KLK10 pre-peptide, the bolded
 sequence (amino acids 34-42) represents the KLK10 pro-peptide, and the remainder of the
 sequence (amino acids 43-276) represents the mature KLK10 protein. In **SEQ ID NO: 2**, the
 aspartic acid at residue 223 of **SEQ ID NO: 1** has been substituted with alanine, and the serine at
 residue 229 of **SEQ ID NO: 1** has been substituted with alanine. Residue 229 is located in the
 conserved catalytic triad region and residue 189 is located in the conserved substrate binding
 pocket region of the KLK10 protein, and the mutations in residues 223 and 229 of **SEQ ID NO: 2**
 (relative to **SEQ ID NO: 1**) resulted in loss of proteolytic activity in the mature protein. (For the
 structural information on KLK10, see Debela et al., *Biol Chem.* 2016;397(12):1251-1264.
 doi:10.1515/hsz-2016-02, incorporated herein by reference).

It is disclosed herein that the KLK10 proteins of **SEQ ID NO: 1** and **SEQ ID NO: 2** exhibit
 anti-atherogenic activity (See, without limitation, Example 1). The corresponding KLK10 pro-
 protein and mature KLK10 protein will have similar biological functions, as the pre-pro-protein and
 pro-protein are processed to mature KLK10 protein when administered to a subject. The pre-pro-
 protein (amino acids 1-276), pro-protein (amino acids 34-276) and mature protein (amino acids 43-
 276) can be linked to another protein, such as, but not limited to, an Fc domain (see below) and
 used in any of the methods disclosed herein.

In some embodiments, the KLK10 protein is at least 90% identical to **SEQ ID NO: 1** or **SEQ ID NO: 2**, such as a KLK10 protein at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to **SEQ ID NO: 1** or **SEQ ID NO: 2**, respectively, wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, and/or atherosclerosis. In more embodiments, the KLK10 protein is at least 90% identical to amino acids 34-276 of **SEQ ID NO: 1** or **SEQ ID NO: 2**, such as a KLK10 protein at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to amino acids 34-276 of **SEQ ID NO: 1** or **SEQ ID NO: 2**, respectively, wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, and/or atherosclerosis. In further embodiments, the KLK10 protein is at least 90% identical to amino acids 43-276 of **SEQ ID NO: 1** or **SEQ ID NO: 2**, such as a KLK10 protein at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to amino acids 43-276 of **SEQ ID NO: 1** or **SEQ ID NO: 2**, respectively, wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, and/or atherosclerosis.

In further embodiments, the KLK10 protein includes at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions in **SEQ ID NO: 1** or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 conservative substitutions in **SEQ ID NO: 2**, wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, atherosclerosis, stroke, peripheral artery disease, and/or myocardial infarction. In yet other embodiments, the KLK10 protein includes at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions in amino acids 34-276 of **SEQ ID NO: 1** or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 conservative substitutions in amino acids 34-276 of **SEQ ID NO: 2**, wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, atherosclerosis, stroke, peripheral artery disease, and/or myocardial infarction. In further embodiments, the KLK10 protein includes at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions in amino acids 43-276 of **SEQ ID NO: 1** or at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 conservative substitutions in amino acids 43-276 of **SEQ ID NO: 2**, wherein the protein retains protective activity against endothelial inflammation, barrier dysfunction, atherosclerosis, stroke, peripheral artery disease, and/or myocardial infarction.

In some embodiments, the KLK10 protein itself, such as a pre-pro-protein, pro-protein or mature form, is used to treat atherosclerosis in a subject. Thus, the therapeutic molecule can consist of a pre-pro-protein KLK10, a pro-protein KLK10, or a mature KLK10.

Exemplary peptides also include derivative peptides that can be one modified by glycosylation, pegylation, phosphorylation or any similar process that retains at least one biological function of the peptide from which it was derived. Peptides of use can also include one or more

non-naturally occurring amino acids. For example, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into peptides. Non-classical amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, alpha-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, gamma-Abu, epsilon-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, beta-alanine, fluoro-amino acids, designer amino acids such as beta-methyl amino acids, Calpha-methyl amino acids, Nalpha-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary). In other specific embodiments, branched versions of the peptides listed herein are provided, such as by substituting one or more amino acids within the sequence with an amino acid or amino acid analog with a free side chain capable of forming a peptide bond with one or more amino acids (and thus capable of forming a "branch"). Cyclical peptides are also contemplated.

Also included are peptide derivatives which are differentially modified during or after synthesis, such as by benzylation, glycosylation, acetylation, phosphorylation, amidation, pegylation, derivatization by known protecting/blocking groups.

The half-life of peptides and proteins in human serum is dictated by several factors, including size, charge, proteolytic sensitivity, nature of their biology, and the turnover rate of proteins they bind. In further embodiments, a fusion protein can be used as the therapeutic molecule. Suitable fusions include any protein that can be used to increase half-life, such as conjugation to a high density lipoprotein, transferrin, albumin, or an Fc domain.

Fc Domain

The present disclosure provides fusion proteins comprising a KLK10 protein (such as a KLK10 pre-pro-protein, a KLK10 pro-protein, or a mature KLK10 protein), and a protein that increases half-life, such as a fragment crystallizable (Fc) domain. These fusion proteins can be recombinantly produced, or the KLK10 protein can be chemically conjugated (including covalent or non-covalent conjugations) to the protein that increases half-life, such as the Fc domain.

In some embodiments, a fusion protein including KLK10 and an Fc domain, or a nucleic acid encoding this fusion protein, is used to treat atherosclerosis. The Fc domain can be monomeric or dimeric. Without being bound by theory, the Fc domain increases the half-life of an immunoglobulin through its unique pH-dependent association with the neonatal Fc receptor (FcRn). For example, after internalization, the Fc domain of IgG can bind to FcRn in

the acidic environment of the endosome, so that the IgG is then cycled onto the cell surface and re-released into circulation. This biological system protects IgG from degradation and results in a long serum half-life. The half-life of a particular type of IgG molecule or its fragments containing FcRn-binding sites in the circulation of, for example, a subject, is represented by the time required for half the quantity administered to the subject to be cleared from the circulation and/or other tissues in the subject. A clearance curve constructed as a function of time for a given IgG is usually biphasic with a rapid alpha-phase that represents an equilibration of the administered IgG molecules between the intra- and extra-vascular space, and a longer beta-phase that represents IgG molecule catabolism in the intravascular space.

In some embodiments, fusion proteins of the present disclosure comprise a KLK10 protein fused to an Fc domain having one or more amino acid substitutions, such as one or more of the amino acid substitutions disclosed herein. In such examples, fusion of the KLK10 protein to the Fc domain increases the *in vivo* half-life of KLK10. In some embodiments, the Fc domain of a disclosed fusion protein has one or more amino acid modifications (i.e., substitutions, deletions, or insertions), for example in amino acid residues identified to be involved in the interaction between the Fc domain and the FcRn receptor.

The Fc domain is constant among wild-type antibodies; the naturally occurring Fc includes the CH2 and CH3 domains, and interacts with neonatal Fc receptor (FcRn), which increases the half-life. In addition, a high affinity interaction that occurs between two CH3 domains makes wild-type Fc a homodimer. Generally, monomeric Fc domains include both a CH2 and a CH3 domain, and are small, stable, and soluble, with minimal to no toxicity. Monomeric forms of Fc domain are available that are, for example, 95%, 96%, 97%, 98% or 99% monomeric. Any of these Fc domains can be fused to a KLK10 protein. An Fc domain can be an IgG Fc, such as an IgG1, IgG2, IgG3 or IgG4 Fc domain. However, in other embodiments, the monomeric Fc is an IgA, IgM, or IgD Fc domain.

The IMGT positions of the CH2 and CH3 domains are provided (see Lefranc et al., *Dev. Comp. Immunol.* 29: 185-203, 2005, herein incorporated by reference) and are also shown in the tables below.

Amino Acid Positions of CH2 Domain Beta Strands

Beta Strand	IMGT Position
A	239-243
B	258-266
C	272-279
C1	281-284
D	288-294
E	300-307
F	318-325

Beta Strand	IMGT Position
G	332-337

Amino Acid Positions of CH3 Domain Beta Strands

Beta Strand	IMGT Position
A'	347-351
B'	362-373
C'	377-383
D'	389-393
E'	404-413
F'	423-428
G'	436-441

As shown in FIGS. 3A-3C of PCT Publication No. WO2009/099961, a CH2 domain comprises six loop regions: Loop 1, Loop 2, Loop 3, Loop A-B, Loop C-D, and Loop E-F. Loops A-B, C-D and E-F are located between beta-strands A and B, C and D, and E and F, respectively. Loops 1, 2, and 3 are located between beta strands B and C, D and E, and F and G, respectively. See Table 1 of PCT Publication No. WO 2009/099961 for the amino acid ranges of the loops in a CH2 domain. Thus, the beta strands and the loop regions of CH2 and CH3 are delineated. The discussion below refers to IMGT position.

Fusions of one or more Fc domains and a therapeutic molecule, such as a KLK10 protein, can increase the half-life of the therapeutic molecule. Increasing the half-life of such therapeutic molecules can reduce the amount and/or frequency of dosing of these molecules. In embodiments of the present disclosure, the *in vivo* half-life of a molecule, such as a KLK10-Fc domain fusion protein, corresponds to the half-life of the molecule in the beta-phase of the relevant clearance curve.

The Fc domain can be monomeric or dimeric, such as homodimeric. Certain embodiments relate to Fc domains that include one or more amino acid modifications relative to a wild-type Fc domain. Modifications that increase the affinity of the Fc domain for the FcRn generally also increase the half-life of the modified Fc domain as compared to the wild-type Fc domain. Such modifications can be made in a monomeric or dimeric Fc domain. One or more Fc domain modifications that increase the affinity of the Fc domain for the FcRn and which increase the half-life of the Fc domain, and thus also of Fc domain fusion proteins, such as an Fc-KLK10 fusion protein, can be made in the CH2, CH3, and/or hinge regions of the Fc domain. Exemplary amino acid modifications that increase the half-life of an Fc domain can be found in U.S. Patent No. 7,658,921 and U.S. Patent No. 9,200,060 which are incorporated herein by reference. An Fc domain useful herein may also include amino acid modifications that disable Fc effector function.

In specific, non-limiting embodiments, the one or more amino acid modifications that disable Fc effector function or increase half-life are made in one or more of residues 234, 235, 252, 254, and/or 256 of an IgG Fc CH2 region, or in analogous residues thereof, as determined by amino acid sequence alignment, in other immunoglobulins. In such examples, the phenylalanine of residue 234 is substituted with an alanine, the leucine of residue 235 is substituted with an alanine, the methionine of residue 252 is substituted with a tyrosine, the serine of residue 254 is substituted with a threonine, and/or the threonine of residue 256 is substituted with a glutamic acid (for example, as in the human IgG4 Fc CH2 region depicted in FIG. 19). The F234A and/or L235A mutations disable Fc effector function, and the M252Y, S254T, and/or T256E mutations increase Fc domain half-life (and thus also increase the half-life of an Fc domain fusion protein, such as a Fc-KLK10 fusion protein).

In some embodiments, the Fc domain is monomeric. The monomeric Fc domains of use in the methods disclosed herein are at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% monomeric. Any one or more amino acid substitutions that significantly reduce or disable the ability of the Fc domain to dimerize can be used, provided that the half-life of a therapeutic molecule (such as KLK10) included in a fusion protein comprising the Fc domain has an increased half-life as compared to the therapeutic molecule alone. The one or more Fc domain modifications that significantly reduce or disable the ability of the Fc domain to dimerize can be made, for example, in the CH3 and/or hinge regions of the Fc domain. Exemplary amino acid modifications that significantly reduce or disable the ability of the Fc domain to dimerize can be found in U.S. Patent No. 9,200,060, which is incorporated herein by reference.

To discourage formation of an Fc domain dimer, such as a homodimer, one or more residues that make up the CH3-CH3 interface can be replaced with a charged amino acid such that interaction between Fc monomers becomes electrostatically unfavorable. For example, a positively-charged amino acid in the interface, such as lysine, arginine, or histidine, can be replaced with a negatively-charged amino acid, such as aspartic acid or glutamic acid, and/or a negatively-charged amino acid in the interface can be replaced with a positively-charged amino acid.

In specific, non-limiting embodiments, the one or more amino acid modifications that discourage Fc domain dimerization are made in one or more of residues 351, 366, 395, 405, and/or 407 of an IgG Fc CH3 region, or in analogous residues thereof, as determined by amino acid sequence alignment, in other immunoglobulins. In such examples, the leucine of residue 351 is substituted with a phenylalanine, the threonine of residue 366 is substituted with an arginine, the proline of residue 395 is substituted with a lysine, the phenylalanine of residue 405 is substituted with an arginine, and/or the tyrosine of residue 407 is substituted with a glutamic acid (for example,

as in the human IgG4 Fc CH3 region depicted in FIG. 19). These mutations significantly reduce or disable CH3-mediated Fc dimerization.

In other specific, non-limiting embodiments, the one or more amino acid modifications that discourage formation of an Fc domain homodimer are made in one or more of residues 226 and/or 229 of an IgG Fc hinge region, or in analogous residues thereof, as determined by amino acid sequence alignment, in other immunoglobulins. In such examples, the cystine of residue 226 is substituted with a serine, and/or the cystine of residue 229 is substituted with a serine (for example, as in the human IgG4 hinge-Fc region depicted in FIG. 19). The C226S and/or C229S mutations significantly reduce or disable hinge-mediated Fc dimerization, see Table 5.

In some embodiments, the fusion protein comprises a KLK10 protein fused to an Fc domain having an amino acid substitution in a) one or more of residues 226 and/or 229 of an Fc hinge region; b) in one or more of residues 234, 235, 252, 254, and/or 256 of an Fc CH2 region; c) in one or more of residues 351, 366, 395, 405, and/or 407 of an Fc CH3 region; or in analogous residues thereof, as determined by amino acid sequence alignment.

In some embodiments, the Fc domain comprises an amino acid substitution at one or more of residues 226, 229, 234, 235, 252, 254, 256, 351, 366, 395, 405, or 407, and wherein the numbering of the amino acids in the Fc domain is IMGT numbering. In other embodiments, the Fc domain comprises at least one amino acid substitution at: a) one or more of residues 226 and 229 of the Fc hinge region; b) one or more of residues 234, 235, 252, 254, and 256 of the Fc CH2 region; or c) one or more of residues 351, 366, 395, 405, and 407 of the Fc CH3 region, wherein the numbering of the amino acids in the Fc domain is IMGT numbering. In more embodiments, the Fc domain comprises an amino acid substitution at one or more of residues C226S, C229S, F234A, L235A, M252Y, S254T, T256E, L351F, T366R, P395K, F405R, or Y407E, and wherein the numbering of the amino acids in the Fc domain is IMGT numbering. In further embodiments, the Fc domain comprises at least one amino acid substitution at: a) one or more of residues C226S and C229S of the Fc hinge region; b) one or more of residues F234A, L235A, M252Y, S254T, and T256E of the Fc CH2 region; or c) one or more of residues L351F, T366R, P395K, F405R, and Y407E of the Fc CH3 region, wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

The monomeric nature of the modified Fc can be assessed by, for example, size exclusion chromatography and/or analytical ultracentrifugation. It is contemplated that modification of a wild-type Fc to produce a substantially monomeric Fc is not limited to IgG Fc but is also applicable to the Fc region of other immunoglobulin subclasses, including IgA, IgE, IgD, and IgM.

Exemplary amino acid sequences of IgG₄ monomeric Fc domains useful in the present disclosure are provided in **SEQ ID NOs: 7-8**:

SEQ ID NO: 7 (an Fc domain of a monomeric Fc-KLK10 fusion protein comprising the following 12 amino acids substitutions: C226S, C229S, F234A, L235A, M252Y, S254T, T256E, L351F, T366R, P395K, F405R, Y407E):

ESKYGPPSPSSPAPEAAGGPSVFLFPPKPKDTLYITREPEVTCVVVDVSQEDPEVQFNWYVD
GVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
GQPREPQVYTFPPSQEEMTKNQVSLRCLVKGFYPSDIAVEWESNGQPENNYKTTKPVLDSDGSFRLESRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLGK

SEQ ID NO: 8 (an Fc domain of a monomeric Fc-KLK10 fusion protein comprising the following 7 amino acids substitutions: C226S, C229S, L351F, T366R, P395K, F405R, Y407E):

ESKYGPPSPSSPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVD
GVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
GQPREPQVYTFPPSQEEMTKNQVSLRCLVKGFYPSDIAVEWESNGQPENNYKTTKPVLDSDGSFRLESRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLGK

In some embodiments, the Fc domain is dimeric. The dimeric Fc domains of use in the methods disclosed herein are at least about 85% dimeric, such as about 90%, about 95%, about 96%, about 97%, about 98%, about 99%, or substantially 100% dimeric. In some examples, fusion proteins comprising a dimeric Fc domain and a therapeutic molecule, such as a KLK10 protein, exhibit increased stability and half-life.

Any one or more amino acid substitutions that significantly increase the ability of the Fc domain to dimerize can be used, provided that the half-life of a therapeutic molecule (such as KLK10) included in a fusion protein comprising the Fc domain has an increased half-life as compared to the therapeutic molecule alone. One or more Fc domain modifications that significantly increase the ability of the Fc domain to dimerize can be made, for example, in the hinge region of the Fc domain.

In specific, non-limiting examples, an amino acid modification is made in residue 228 of an Fc hinge region, or in analogous residues thereof, as determined by amino acid sequence alignment, in other immunoglobulins. In such examples, the serine of residue 228 is substituted with a proline (for example, as in the human IgG₄ hinge-Fc region depicted in FIG. 19). The S228P mutation increases stability of hinge-mediated Fc dimerization.

Dimerization may be measured by one or more techniques known in the art, including size exclusion chromatography, analytical ultracentrifugation, dynamic light scattering, and/or native PAGE. It is contemplated that modification of a wild-type Fc to enhance Fc dimerization is not limited to IgG Fc but is also applicable to the Fc region of other immunoglobulin subclasses, including IgA, IgE, IgD, and IgM. Virtually any molecule that contains an Fc domain may comprise a dimeric Fc domain of the present invention.

In some embodiments, the fusion protein comprises a KLK10 protein fused to an Fc domain having an amino acid substitution in residue 228 of the Fc hinge region; in one or more of residues 234, 235, 252, 254, and/or 256 of the Fc CH2 region; or in analogous residues thereof, as determined by amino acid sequence alignment, in other immunoglobulins.

In more embodiments, the Fc domain comprises an amino acid substitution at one or more of residues 228, 234, 235, 252, 254, and 256, and wherein the numbering of the amino acids in the Fc domain is IMGT numbering. In further embodiments, the Fc domain comprises an amino acid substitution at least one of: a) residue 228 of the Fc hinge region; or b) one or more of residues 234, 235, 252, 254, and 256 of the Fc CH2 region, wherein the numbering of the amino acids in the Fc domain is IMGT numbering. In yet other embodiments, the Fc domain comprises one or more of S228P, F234A, L235A, M252Y, S254T, and T256E of the Fc region, wherein the numbering of the amino acids in the Fc domain is IMGT numbering. In some embodiments, the Fc domain comprises at least one of the following substitutions: a) S228P of the Fc hinge region; or b) one or more of F234A, L235A, M252Y, S254T, and T256E of the Fc CH2 region, wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

Exemplary amino acid sequences of IgG₄ dimeric Fc domains useful in the present disclosure are provided in **SEQ ID NOs: 9-10**:

SEQ ID NO: 9 (an Fc domain of a dimeric Fc-KLK10 fusion protein comprising the following 7 amino acids substitutions: S228P, F234A, L235A, M252Y, S254T, T256E):
 ESKYGPPCPPCPAPEAAGGPSVFLFPPKPKDTLYITREPEVTCVVVDVSQEDPEVQFNWYV
 DGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKA
 KGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS
 DGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

SEQ ID NO: 10 (an Fc domain of a dimeric Fc-KLK10 fusion protein comprising the following amino acids substitution: S228P):

ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVD
 GVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
 GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSD
 GSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLGLGK

5

In some embodiments, the fusion protein comprises an Fc domain having at least 90% sequence identity to an amino acid sequence set forth in any one of **SEQ ID NOs: 7-10**, such as at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical to an amino acid sequence of **SEQ ID NOs: 7-10**, wherein the fusion of KLK10 to the Fc domain increases the *in vivo* half-life of KLK10, and wherein the KLK10 retains protective activity against endothelial inflammation, barrier dysfunction, and/or atherosclerosis. In further embodiments, the Fc domain of the fusion protein includes at most 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 conservative substitutions any one of **SEQ ID NOs: 7-10**. Exemplary fusion proteins (comprising a KLK10 and an Fc domain) that can be used in the present disclosure are set forth in **SEQ ID NOs: 11-14** as follows.

15

SEQ ID NO: 11 (a monomeric Fc-KLK10 fusion protein, wherein the Fc domain is **SEQ ID NO: 7**):

MRAPHLHLAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDP EAYGSPCARGSQPW
 QVSLFNGLSFHCAGVLVDQSWVLTAAHCGNKPLWARVGDDHLLLLQGEQLRRTTTRSVVH
 20 PKYHQGSGPILPRRTDEHDLMLLKLARPVVLGPRVRALQLPYRCAQPGDQCQVAGWGTT
 AARRVKYNKGLTCSSITILSPKECEVFYPGVVTNNMICAGLDRGQDPCQSDSGGPLVCDET
 LQGILSWG VYPCGSAQH PAVYTQICKYMSWINKVIRS NESKYGPPSPSSPAPEAAGGPSVFL
 FPPKPKDTLYITREPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRV
 VSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTFPPS QEEMTKNQ
 25 VSLRCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDGSFRLESRLTVDKSRWQEGNV
 FSCSVMHEALHNHYTQKSLSLGLGK

SEQ ID NO: 12 (a monomeric Fc-KLK10 fusion protein, wherein the Fc domain is **SEQ ID NO: 8**):

MRAPHLHLAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDP EAYGSPCARGSQPW
 QVSLFNGLSFHCAGVLVDQSWVLTAAHCGNKPLWARVGDDHLLLLQGEQLRRTTTRSVVH
 30 PKYHQGSGPILPRRTDEHDLMLLKLARPVVLGPRVRALQLPYRCAQPGDQCQVAGWGTT
 AARRVKYNKGLTCSSITILSPKECEVFYPGVVTNNMICAGLDRGQDPCQSDSGGPLVCDET
 LQGILSWG VYPCGSAQH PAVYTQICKYMSWINKVIRS NESKYGPPSPSSPAPEFLGGPSVFL

FPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRV
 VSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTFPPSQEEMTKNQ
 VSLRCLVKGFYPSDIAVEWESNGQPENNYKTTKPVLDSDGSFRLESRLTVDKSRWQEGNV
 FSCSVMHEALHNHYTQKSLSLGLGK

5

SEQ ID NO: 13 (a dimeric Fc-KLK10 fusion protein, wherein the Fc domain is **SEQ ID NO: 9**):

MRAPHLHLAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDPEDAYGSPCARGSQPW
 QVSLFNGLSFHCAGVLVDQSWVLTAHCGNKPLWARVGDDHLLLLQGEQLRRTTRSVVH
 10 PKYHQGSGPILPRRTDEHDLMLLKLARPVVLGPRVRALQLPYRCAQPGDQCQVAGWGTT
 AARRVKYNKGLTCSSITILSPKECEVFYPGVVTNNMICAGLDRGQDPCQSDSGGPLVCDET
 LQGILSWGVPYPCGSAQHAPVYTQICKYMSWINKVIRSNEISKYGPCCPPCAPEAAGGPSVF
 LFPPKPKDTLYITREPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYR
 VVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKN
 15 QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGN
 VFSCSVMHEALHNHYTQKSLSLGLGK

SEQ ID NO: 14 (a dimeric Fc-KLK10 fusion protein, wherein the Fc domain is **SEQ ID NO: 10**):

MRAPHLHLAASGARALAKLLPLLMAQLWAAEAALLPQNDTRLDPEDAYGSPCARGSQPW
 QVSLFNGLSFHCAGVLVDQSWVLTAHCGNKPLWARVGDDHLLLLQGEQLRRTTRSVVH
 PKYHQGSGPILPRRTDEHDLMLLKLARPVVLGPRVRALQLPYRCAQPGDQCQVAGWGTT
 AARRVKYNKGLTCSSITILSPKECEVFYPGVVTNNMICAGLDRGQDPCQSDSGGPLVCDET
 LQGILSWGVPYPCGSAQHAPVYTQICKYMSWINKVIRSNEISKYGPCCPPCAPEFLGGPSVFL
 25 FPPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRV
 VSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ
 VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSRLTVDKSRWQEGNVF
 SCSVMHEALHNHYTQKSLSLGLGK

30 The Fc domains disclosed herein can be of the IgG4 subclass of IgGs but may also be any other IgG subclasses of given animals. For example, in humans, the IgG class includes IgG1, IgG2, IgG3, and IgG4, and mouse IgG includes IgG1, IgG2a, IgG2b, IgG2c and IgG3. It is known that certain IgG sub-classes, for example, mouse IgG2b and IgG2c, have higher clearance rates than, for example, IgG1. Thus, when using IgG subclasses other than IgG1, it may be advantageous to

substitute one or more of the residues, particularly in the CH2 and CH3 domains, that differ from the IgG1 sequence with those of IgG1, thereby increasing the *in vivo* half-life of the other types of IgG.

The Fc domains (and other proteins) used herein may be from any animal including birds and mammals. Preferably, the Fc domains are human, rodent (e.g., mouse or rat), donkey, sheep, rabbit, goat, guinea pig, camel, horse, or chicken. As used herein, human Fc domains include Fc domains having the amino acid sequence of a human Fc domain and include those isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins.

Amino acid modifications can be made by any appropriate method. For example, amino acid substitutions, deletions, and insertions may be accomplished using any well-known PCR-based technique. Amino acid substitutions may be made by site directed mutagenesis. Mutants that result in increased affinity for FcRn and increased *in vivo* half-life may readily be screened using well-known and routine assays.

Mutagenesis may be performed in accordance with any techniques known in the art including, but not limited to, synthesizing an oligonucleotide having one or more modifications within the sequence of the Fc domain (such as the hinge, CH2 and/or CH3 regions) to be modified. Site-specific mutagenesis allows for production of mutants through the use of specific oligonucleotide sequences that encode the DNA sequence of the one or more desired mutations, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to about 75 nucleotides or more in length is preferred, with about 10 to about 25 or more residues on both sides of the junction of the sequence being altered. A number of such primers introducing a variety of different mutations at one or more positions may be used to generate a library of mutants.

The technique of site-specific mutagenesis is well known in the art, as exemplified by various publications (*See, e.g., Kunkel et al., Methods Enzymol.*, 154:367-82, 1987, which is hereby incorporated by reference in its entirety). In general, site-directed mutagenesis is performed by first obtaining a single-stranded vector or melting apart two strands of a double stranded vector that includes within its sequence a DNA sequence encoding the desired peptide. An oligo-nucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector and subjected to DNA polymerizing enzymes, such as T7 DNA polymerase, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the

second strand bears the desired mutation. This heteroduplex vector is then used to transform or transfect appropriate cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement. The technique typically employs a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. Double stranded plasmids are also routinely employed in site directed mutagenesis, thus eliminating the step of transferring the gene of interest from a plasmid to a phage.

Alternatively, the use of PCR with commercially available thermostable enzymes, such as Taq DNA polymerase, may be used to incorporate a mutagenic oligonucleotide primer into an amplified DNA fragment that can then be cloned into an appropriate cloning or expression vector (See, e.g., Tamie et al., *Nucleic Acids Res.*, 18(6):1656, 1987, and Upender et al., *Biotechniques*, 18(1):29-30, 32, 1995, which are hereby incorporated by reference in their entirety). PCR employing a thermo-stable ligase in addition to a thermostable polymerase may also be used to incorporate a phosphorylated mutagenic oligonucleotide into an amplified DNA fragment that may then be cloned into an appropriate cloning or expression vector (See, e.g., Michael, *Biotechniques*, 16(3):410-2, 1994, which is hereby incorporated by reference in its entirety).

Other methods known to those of ordinary skill in art of producing sequence variants of the Fc domain of an antibody can be used. For example, recombinant vectors encoding the amino acid sequence of the constant domain of an antibody or a fragment thereof may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

Nucleic Acid Molecules

Nucleic acid molecules encoding a KLK10 protein, or a fusion protein including a KLK10 protein, such as a fusion protein including a KLK10 protein and an Fc domain, can readily be produced by one of skill in the art using the amino acid sequences provided herein and the genetic code. Optionally, a nucleic acid molecule encoding a KLK10 sequence can also include a His-tag. An exemplary nucleotide sequence encoding the human KLK10 gene is provided in **SEQ ID NO: 5** (GENBANK® Accession No. NC_000019.10 (c51020175-51012739), incorporated by reference herein as of September 8, 2020, and Ensembl Gene ID: ENSG00000129451, incorporated by reference herein as of September 8, 2020). In some examples, the method includes administering a nucleic acid molecule encoding a KLK10 protein (such as a nucleic acid molecule encoding a KLK10 pre-pro-protein, a KLK10 pro-protein, or a mature KLK10 protein).

An exemplary nucleic acid encoding human KLK10 is:

ACAATCTCCCTTTTCAAGCCAGCCTCTGTCCCTCCTACTCAACCTGCTTTATCTCTAGGC
CTTCCTCCTTCCTCTTCCACAGTCTGGCTTCTCACATTGTCACTCTAGCAGATCCTGGCC
ATGAGAGCTCCGCACCTCCACCTCTCCGCCGCTCTGGCGCCCGGGCTCTGGCGAAGC
TGCTGCCGCTGCTGATGGCGCAACTCTGGGCGCGCAGAGGCGGCGCTGCTCCCCAAAA
5 CGACACGCGCTTGACCCCCGAAGCCTATGGCTCCCCGTGCGCGCGCGGCTCGCAGCCC
TGGCAGGTCTCGCTCTTCAACGGCCTCTCGTTCCACTGCGCGGGTGTCTGGTGGACCA
GAGTTGGGTGCTGACGGCCGCGCACTGCGGAAACAAGCCACTGTGGGCTCGAGTAGG
GGATGACCACCTGCTGCTTCTTCAGGGAGAGCAGCTCCGCCGGACCACTCGCTCTGTT
GTCCATCCCAAGTACCACCAGGGCTCAGGCCCCATCCTGCCAAGGCGAACGGATGAGC
10 ACGATCTCATGTTGCTGAAGCTGGCCAGGCCCGTAGTGCTGGGGGCCCCGCGTCCGGGC
CCTGCAGCTTCCCTACCGCTGTGCTCAGCCCGGAGACCAGTGCCAGGTTGCTGGCTGG
GGCACCACGGCCGCCCGGAGAGTGAAGTACAACAAGGGCCTGACCTGCTCCAGCATC
ACTATCCTGAGCCCTAAAGAGTGTGAGGTCTTCTACCCTGGCGTGGTCACCAACAACA
TGATATGTGCTGGACTGGACCGGGGCCAGGACCCTTGCCAGAGTGACTCTGGAGGCCC
15 CCTGGTCTGTGACGAGACCCTCCAAGGCATCCTCTCGTGGGGTGTTTACCCCTGTGGCT
CTGCCCAGCATCCAGCTGTCTACACCCAGATCTGCAAATACATGTCTGGATCAATAA
AGTCATACGCTCCAACCTGATCCAGATGCTACGCTCCAGCTGATCCAGATGTTATGCTCC
TGCTGATCCAGATGCCAGAGGCTCCATCGTCCATCCTCTTCCCTCCCCAGTCGGCTGAA
CTCTCCCCTTGTCTGCACTGTTCAAACCTCTGCCGCCCTCCACACCTCTAAACATCTCCC
20 CTCTCACCTCATTTCCCCACCTATCCCCATTCTCTGCCTGTACTGAAGCTGAAATGCAG
GAAGTGGTGGCAAAGGTTTATTCCAGAGAAGCCAGGAAGCCGGTCATCACCCAGCCTC
TGAGAGCAGTTACTGGGGTCACCCAACCTGACTTCCTCTGCCACTCCCTGCTGTGTGAC
TTTGGGCAAGCCAAGTGCCCTCTCTGAACCTCAGTTTCTCATCTGCAAAATGGGAACA
ATGACGTGCCTACCTCTTAGACATGTTGTGAGGAGACTATGATATAACATGTGTATGTA
25 AATCTTCATGGTGATTGTTCATGTAAGGCTTAACACAGTGGGTGGTGAGTTCTGACTAA
AGGTTACCTGTTGTCGTGATCTGACCACGTCCCGGTGAAAGCGTGTGTCCAGGGAAGA
AGTGCACAGGGTAGCCCCCAGTCCCAACCTTCCATCCCCAACCTTAGGGATGATGGA
AGAATCATTTTCTCACCTAGTTCCAAGTCCCAGGAAACACCTTTTAACCACTTCCTT
CTCATCTCCCCTGTTTCCCCTTCTGGTTCCACCCAACACCAGTTCCTCCGAGCTAGG
30 CTGGCCCTGAGTCATTAGCACCTTCTCTGCCTTCATGAGGCCACTGAACTCAAGGGACC
TCACCTTGCTTCTATCCCAGCCTCTAAGACCAGAGGGCCGAGGGGGTAGTGAAGATTG
GGAAACCCTTGCCACCTCAACTGCCAGCTTGTGCCAAGAAACCTCCCTCTGACATTTA
GGGGAAAATCTTTGGTTTGTCTGTTATTAATTGGCTGTGAGATTTTCGTCTGAAAAC
TGGGAGGAGGAATTGTTTGATTCTCCCTGAAATTGGGGGAGGGAAGGGGAGTTAATAC
35 ACAGAATCCAGGTAAGGCTAATAGAAGCTTCAGTGTCCGCTGGGTGTGGTGGCTCACG
CCTGTAATCCTAGCACTTTGGGAGGCCGAGGAAAGCAGATCATCTGAGGTCAGGTGTT
CGAGACCAGTCTGGCCAAAATGGTGAAATCCCATCTCTACTGAAAATACAAAACCTAG
CCAGGCATGGTGGCAGGCACCTATAATCCCATCTACTTGGGAGGCTGAGGCAGAAGAA
TCACTTGAACCCGGGGGGCAGAGGTTGCAGTGAGCCAAGATTGCACCATTTGCACTCCA
40 TCCAGCCTGGACAAAAGAGCAAACTCCATCTCAAAAAAAGAAGAAGTTTGAGTGTCT
CAGCTTATCCTGAACTTTGAAGCAGTAACATAAGCTAGCTGAATATAGGCACAACAGA
AATTTCCCATTAAGCACGGGGTTTTTGTGTTGTTTGTGTTTGTCTTTCAGTTACCAATTTAT
GGAGCATCTATTATGTGCCAGGCCAGTGCTGGGTGCTGGGGACATACGTAGGGGTGA
TCACAAGGTCCCCCACCCTGCAGAGCCACAGGAGGTTGATGTACAAGGTCTGAGCAC
45 ACAGCTCCCTTCCGTGGCCTCTTTCCCATTCTGCCCCCATTAGCACACGCAGGAAATGT

CAGACAGGCGTATCAGCTGGGTTTGTTCATCCAAAGACCAGAAGCGAGGTCGGTGGAA
 ACTTGAAAACCTCGATTTCATTATTAAAGGCAACTCACCCGTCCCTTTAGTCACTCCACAA
 TGTTTATCGAGCCACGTCTTATGCCAGGCCTCAAAGATGAATCAGACCAGGCACTACC
 CTCAAGGAGCTGCTAGTTAGGTCAGGGAGACAGGCCAGTCTTACATTCCTGTCATTCA
 5 GGTCTCCACTCAAAAGTCACCTCCTCCGGGAGGCCTTCCTGAATTGCCCAGGCTATAG
 GAGTCCACTTTTGGTCATCCAGTCCCGATGCTCTGTTTTTTGTTTGTGTTTTTTTTTTT
 AATAGCACCTATTATACCTGAAATTAATAAAAAAAAAAAAAAAAAAAAAA (SEQ ID NO: 6; see
 GenBank Accession No. NM_001077500.1, incorporated herein by reference as of September 8,
 2020, which encodes the KLK10 protein of SEQ ID NO: 1, GenBank Accession No.
 10 NP_001070968.1, September 8, 2020, also incorporated by reference).

Nucleic acid sequences encoding the KLK10 protein or the fusion protein can be prepared
 by any suitable method including, for example, cloning of appropriate sequences or by direct
 chemical synthesis by methods such as the phosphotriester method of Narang et al., *Meth. Enzymol.*
 15 68:90-99, 1979; the phosphodiester method of Brown et al., *Meth. Enzymol.* 68:109-151, 1979; the
 diethylphosphoramidite method of Beaucage et al., *Tetra. Lett.* 22:1859-1862, 1981; the solid phase
 phosphoramidite triester method described by Beaucage & Caruthers, *Tetra. Letts.* 22(20):1859-
 1862, 1981, for example, using an automated synthesizer as described in, for example, Needham-
 VanDevanter et al., *Nucl. Acids Res.* 12:6159-6168, 1984 and the solid support method of U.S.
 20 Patent No. 4,458,066. Chemical synthesis produces a single-strand (ss) oligonucleotide, which can
 be converted into double-strand (ds) DNA by hybridization with a complementary sequence or by
 polymerization with a DNA polymerase using the single strand as a template. Exemplary nucleic
 acids that include sequences encoding a KLK10 protein or the fusion protein can be prepared by
 cloning techniques.

25 A nucleic acid molecule encoding a KLK10 protein or the fusion protein can be cloned or
 amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain
 reaction (LCR), the transcription-based amplification system (TAS), the self-sustained sequence
 replication system (3SR), and the Q β replicase amplification system (QB). For example, a
 polynucleotide encoding the protein can be isolated by a polymerase chain reaction of cDNA using
 30 primers based on the DNA sequence of the molecule. A wide variety of cloning and *in vitro*
 amplification methodologies are well-known to persons skilled in the art. PCR methods are
 described in, for example, U.S. Patent No. 4,683,195; Mullis et al., *Cold Spring Harbor Symp.*
Quant. Biol. 51:263, 1987; and Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989).
 Polynucleotides also can be isolated by screening genomic or cDNA libraries with probes selected
 35 from the sequences of the desired polynucleotide under stringent hybridization conditions.

Typically, a polynucleotide sequence encoding a KLK10 protein or the fusion protein is
 operably linked to transcriptional control sequences including, for example a promoter and a

polyadenylation signal. Any promoter can be used that is a polynucleotide sequence recognized by the transcriptional machinery of the host cell (or introduced synthetic machinery) that is involved in the initiation of transcription. A polyadenylation signal is a polynucleotide sequence that directs the addition of a series of nucleotides on the end of the mRNA transcript for proper processing and trafficking of the transcript out of the nucleus into the cytoplasm for translation.

Exemplary promoters include viral promoters, such as cytomegalovirus immediate early gene promoter ("CMV"), herpes simplex virus thymidine kinase ("tk"), SV40 early transcription unit, polyoma, retroviruses, papilloma virus, hepatitis B virus, and human and simian immunodeficiency viruses. Other promoters include promoters isolated from mammalian genes, such as the immunoglobulin heavy chain, immunoglobulin light chain, T cell receptor, HLA DQ α and DQ β , β -interferon, interleukin-2, interleukin-2 receptor, MHC class II, HLA-DR α , β -actin, muscle creatine kinase, prealbumin (transthyretin), elastase I, metallothionein, collagenase, albumin, fetoprotein, β -globin, c-fos, c-HA-ras, neural cell adhesion molecule (NCAM), α 1-antitrypsin, H2B (TH2B) histone, type I collagen, glucose-regulated proteins (GRP94 and GRP78), rat growth hormone, human serum amyloid A (SAA), troponin I (TNI), platelet-derived growth factor, and dystrophin, as well as promoters specific for retinal cells.

The promoter can be either inducible or constitutive. An inducible promoter is a promoter that is inactive or exhibits low activity except in the presence of an inducer substance. Additional examples of promoters include, but are not limited to, MT II, MMTV, collagenase, stromelysin, SV40, murine MX gene, α -2-macroglobulin, MHC class I gene h-2kb, HSP70, proliferin, tetracycline inducible, tumor necrosis factor, or thyroid stimulating hormone gene promoter. One example of an inducible promoter is the interferon inducible ISG54 promoter (see Bluysen et al., *Proc. Natl Acad. Sci.* 92: 5645-5649, 1995, herein incorporated by reference). In some embodiments, the promoter is a constitutive promoter that results in high levels of transcription upon introduction into a host cell in the absence of additional factors.

Optionally, transcription control sequences include one or more enhancer elements, which are binding recognition sites for one or more transcription factors that increase transcription above that observed for a minimal promoter alone, and also be operably linked to the polynucleotide encoding the promoter and/or the nucleic acid molecule encoding the KLK10 protein or the fusion protein. With regard to the nucleic acid molecule encoding the CR KLK10 protein or the fusion protein, introns can also be included that help stabilize mRNA and increase expression.

In some embodiments of the compositions and methods described herein, a nucleic acid sequence that encodes a KLK10 protein or a fusion protein is incorporated into a vector capable of expression in a host cell, using established molecular biology procedures. For example, nucleic

acids, such as cDNAs, that encode a KLK10 protein or a fusion protein can be manipulated with standard procedures, such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence-alteration via single-stranded bacteriophage intermediate, or use of specific oligonucleotides in combination with PCR or other *in vitro* amplification.

Non-limiting examples of procedures sufficient to guide one of ordinary skill in the art through the production of a vector capable of expression in a host cell that includes a promoter, and/or a polynucleotide sequence encoding a KLK10 protein or a fusion protein can be found, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 3d ed., Cold Spring Harbor Press, 2001; Ausubel et al., *Current Protocols in Molecular Biology*, Greene Publishing Associates, 1992 (and Supplements to 2003); and Ausubel et al., *Short Protocols in Molecular Biology: A Compendium of Methods from Current Protocols in Molecular Biology*, 4th ed., Wiley & Sons, 1999.

It may be desirable to include a polyadenylation signal to effect proper termination and polyadenylation of the gene transcript. Exemplary polyadenylation signals have been isolated from beta globin, bovine growth hormone, SV40, and the herpes simplex virus thymidine kinase genes.

The disclosed nucleic acid molecules can be included in a nanodispersion system. *See, e.g.*, U.S. Pat. No. 6,780,324; U.S. Pat. Publication No. 2009/0175953. For example, a nanodispersion system includes a biologically active agent and a dispersing agent (such as a polymer, copolymer, or low molecular weight surfactant). Exemplary polymers or copolymers include polyvinylpyrrolidone (PVP), poly(D,L-lactic acid) (PLA), poly(D,L-lactic-co-glycolic acid (PLGA), and poly(ethylene glycol). Exemplary low molecular weight surfactants include sodium dodecyl sulfate, hexadecyl pyridinium chloride, polysorbates, sorbitans, poly(oxyethylene) alkyl ethers, poly(oxyethylene) alkyl esters, and combinations thereof. In one example, the nanodispersion system includes PVP and ODP or a variant thereof (such as 80/20 w/w). In some examples, the nanodispersion is prepared using the solvent evaporation method. *See, e.g.*, Kanaze et al., *Drug Dev. Indus. Pharm.* 36:292-301, 2010; Kanaze et al., *J. Appl. Polymer Sci.* 102:460-471, 2006.

Dendrimers are synthetic three-dimensional macromolecules that are prepared in a step-wise fashion from simple branched monomer units, the nature and functionality of which can be easily controlled and varied. Dendrimers consist of an initiator core, surrounded by a layer of a selected polymer that is grafted to the core, forming a branched macromolecular complex. Dendrimers are typically produced using polymers such as poly(amidoamine) or poly(L-lysine). A dendrimer can be synthesized from the repeated addition of building blocks to a multifunctional core (divergent approach to synthesis), or towards a multifunctional core (convergent approach to

synthesis) and each addition of a three-dimensional shell of building blocks leads to the formation of a higher generation of the dendrimers. Polypropylenimine dendrimers contain 100% protonable nitrogens and up to 64 terminal amino groups. Protonable groups are usually amine groups which are able to accept protons at neutral pH. For nucleic acid molecules, dendrimers can be formed from polyamidoamine and phosphorous containing compounds with a mixture of amine/ amide or N-P(O₂)S as the conjugating units. Dendrimers of use for delivery of nucleic acid molecules is disclosed, for example, in PCT Publication No. 2003/033027, imported herein by reference.

The polynucleotides encoding the KLK10 protein, or the fusion protein, include a recombinant DNA which is incorporated into a vector in an autonomously replicating plasmid or virus or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (such as a cDNA) independent of other sequences. Viral vectors that include the KLK10 protein or the fusion protein can also be prepared. Numerous viral vectors are known in the art, including polyoma; SV40 (Madzak et al., 1992, *J. Gen. Virol.*, 73:1533-1536); adenovirus (Berkner, 1992, *Cur. Top. Microbiol. Immunol.*, 158:39-6; Berliner et al., 1988, *Bio Techniques*, 6:616-629; Gorziglia et al., 1992, *J. Virol.*, 66:4407-4412; Quantin et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:2581-2584; Rosenfeld et al., 1992, *Cell*, 68:143-155; Wilkinson et al., 1992, *Nucl. Acids Res.*, 20:2233-2239; Stratford-Perricaudet et al., 1990, *Hum. Gene Ther.*, 1:241-256); vaccinia virus (Mackett et al., 1992, *Biotechnology*, 24:495-499); adeno-associated virus (Muzyczka, 1992, *Curr. Top. Microbiol. Immunol.*, 158:91-123; On et al., 1990, *Gene*, 89:279-282); herpes viruses, including HSV and EBV (Margolskee, 1992, *Curr. Top. Microbiol. Immunol.*, 158:67-90; Johnson et al., 1992, *J. Virol.*, 66:2952-2965; Fink et al., 1992, *Hum. Gene Ther.* 3:11-19; Breakfield et al., 1987, *Mol. Neurobiol.*, 1:337-371; Fresse et al., 1990, *Biochem. Pharmacol.*, 40:2189-2199); Sindbis viruses (H. Herweijer et al., 1995, *Human Gene Therapy* 6:1161-1167; U.S. Pat. Nos. 5,091,309 and 5,2217,879); alphaviruses (S. Schlesinger, 1993, *Trends Biotechnol.* 11:18-22; I. Frolov et al., 1996, *Proc. Natl. Acad. Sci. USA* 93:11371-11377); and retroviruses of avian (Brandyopadhyay et al., 1984, *Mol. Cell Biol.*, 4:749-754; Petropoulos et al., 1992, *J. Virol.*, 66:3391-3397), murine (Miller, 1992, *Curr. Top. Microbiol. Immunol.*, 158:1-24; Miller et al., 1985, *Mol. Cell Biol.*, 5:431-437; Sorge et al., 1984, *Mol. Cell Biol.*, 4:1730-1737; Mann et al., 1985, *J. Virol.*, 54:401-407), and human origin (Page et al., 1990, *J. Virol.*, 64:5370-5276; Buchschalcher et al., 1992, *J. Virol.*, 66:2731-2739). Baculovirus (*Autographa californica* multinuclear polyhedrosis virus; AcMNPV) vectors are also known in the art and may be obtained from commercial sources (such as PharMingen, San Diego, Calif.; Protein Sciences Corp., Meriden, Conn.; Stratagene, La Jolla, Calif.).

Thus, in one embodiment, the nucleic acid molecule encoding the KLK10 protein or the

fusion protein, is included in a viral vector. Suitable vectors include retrovirus vectors, orthopox vectors, avipox vectors, fowlpox vectors, capripox vectors, suipox vectors, adenoviral vectors, herpes virus vectors, alpha virus vectors, baculovirus vectors, Sindbis virus vectors, vaccinia virus vectors, lentivirus vectors, and poliovirus vectors. Specific exemplary vectors are poxvirus vectors, such as vaccinia virus, fowlpox virus and a highly attenuated vaccinia virus (MVA), adenovirus, baculovirus, yeast, and the like. Adeno-associated virus vectors (AAV) are disclosed in additional detail below and are of use in the disclosed methods.

AAV Vectors

Disclosed herein are methods and compositions that include one or more vectors, such as a viral vector, such as a retroviral vector or an adenoviral vector, or an AAV vector that includes a promoter operably linked to a nucleic acid molecule including a KLK10 protein or a fusion protein that includes the KLK10 protein. Defective viruses, that entirely or almost entirely lack viral genes, can be used. Use of defective viral vectors allows for administration to specific cells without concern that the vector can infect other cells. The adenovirus vectors of use include replication competent, replication deficient, or gutless forms thereof. The AAV vectors of use are replication deficient. Without being bound by theory, adenovirus vectors are known to exhibit strong expression *in vitro*, excellent titer, and the ability to transduce dividing and non-dividing cells *in vivo* (Hitt et al., *Adv. in Virus Res.* 55:479-505, 2000). When used *in vivo* these vectors lead to strong but transient gene expression due to immune responses elicited to the vector backbone. In some non-limiting examples, a vector of use is a defective AAV vector (Gonçalves, *Virol J.*, 2:43, 2005; Rolling & Samulski, *Mol. Biotech.*, 3:9-15, 1995).

Recombinant AAV vectors are capable of directing expression and production of the selected transgenic products in targeted cells. Thus, the recombinant vectors comprise at least all of the sequences of AAV essential for encapsidation and the physical structures for infection of target cells.

AAV belongs to the family *Parvoviridae* and the genus *Dependovirus*. AAV is a small, non-enveloped virus that packages a linear, single-stranded DNA genome. Both sense and antisense strands of AAV DNA are packaged into AAV capsids with equal frequency. In some embodiments, the AAV DNA includes a nucleic acid including a recombinant *CRX* promoter, as disclosed herein, operably linked to a nucleic acid molecule encoding a CRX protein, such as a human CRX protein. Further provided are recombinant vectors, such as recombinant adenovirus vectors and recombinant adeno-associated virus (rAAV) vectors comprising a nucleic acid molecule(s) disclosed herein. In some embodiments, the AAV is rAAV8, and/or AAV2. However,

the AAV serotype can be any other suitable AAV serotype, such as AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAV10, AAV11 or AAV12, or a hybrid of two or more AAV serotypes. In some embodiments, the AAV is an AAV2, including by not limited to AAV2Q and other rAAV2-based capsid mutants, such as Y272F, Y444F, Y500F, Y730F, T491V (termed “QuadYF+TV”), see Lipinski et al, Hum Gene Ther 26(11):767-76. doi: 10.1089/hum.2015.097. Epub 2015 Sep 29, incorporated herein by reference.

The AAV genome is characterized by two inverted terminal repeats (ITRs) that flank two open reading frames (ORFs). In the AAV2 genome, for example, the first 125 nucleotides of the ITR are a palindrome, which folds upon itself to maximize base pairing and forms a T-shaped hairpin structure. The other 20 bases of the ITR, called the D sequence, remain unpaired. The ITRs are *cis*-acting sequences important for AAV DNA replication; the ITR is the origin of replication and serves as a primer for second-strand synthesis by DNA polymerase. The double-stranded DNA formed during this synthesis, which is called replicating-form monomer, is used for a second round of self-priming replication and forms a replicating-form dimer. These double-stranded intermediates are processed via a strand displacement mechanism, resulting in single-stranded DNA used for packaging and double-stranded DNA used for transcription. Located within the ITR are the Rep binding elements and a terminal resolution site (TRS). These features are used by the viral regulatory protein Rep during AAV replication to process the double-stranded intermediates. In addition to their role in AAV replication, the ITR is also essential for AAV genome packaging, transcription, negative regulation under non-permissive conditions, and site-specific integration (Daya and Berns, *Clin Microbiol Rev* 21(4):583-593, 2008). In some embodiments, these elements are included in the AAV vector.

The left ORF of AAV contains the Rep gene, which encodes four proteins – Rep78, Rep 68, Rep52 and Rep40. The right ORF contains the Cap gene, which produces three viral capsid proteins (VP1, VP2 and VP3). The AAV capsid contains 60 viral capsid proteins arranged into an icosahedral symmetry. VP1, VP2 and VP3 are present in a 1:1:10 molar ratio (Daya and Berns, *Clin Microbiol Rev* 21(4):583-593, 2008). In some embodiments, these elements are included in the AAV vector.

AAV vectors can be used for gene therapy. Exemplary AAV of use are AAV2, AAV5, AAV6, AAV8 and AAV9. Adeno-associated viruses AAV2 and AAV8 are capable of transducing cells in the retina. Thus, any of a rAAV2 or rAAV8 vector can be used in the methods disclosed herein. However, rAAV6 and rAAV9 vectors are also of use.

Although AAV infects humans and some other primate species, it is not known to cause disease and elicits a very mild immune response. Gene therapy vectors that utilize AAV can infect

both dividing and quiescent cells and persist in an extrachromosomal state without integrating into the genome of the host cell. AAV2 preferentially infects cells of the human retina. Because of the advantageous features of AAV, the present disclosure contemplates the use of an rAAV for the methods disclosed herein.

5 AAV possesses several additional desirable features for therapy, including the ability to bind and enter target cells, enter the nucleus, the ability to be expressed in the nucleus for a prolonged period of time, and low toxicity. AAV can be used to transfect cells, and suitable vector are known in the art, see for example, U.S. Published Patent Application No. 2014/0037585, incorporated herein by reference. Methods for producing rAAV suitable for gene therapy are well
10 known in the art (see, for example, U.S. Published Patent Application Nos. 2012/0100606; 2012/0135515; 2011/0229971; and 2013/0072548; and Ghosh et al., *Gene Ther* 13(4):321-329, 2006), and can be utilized with the methods disclosed herein.

In some embodiments, the vector is a rAAV8 vector, a rAAV2 vector, a rAAV9 vector. In a specific non-limiting example, the vector is an AAV8 vector. AAV8 vectors are disclosed, for
15 example, in U.S. Patent No. 8,692,332, which is incorporated by reference herein. The location and sequence of the capsid, rep 68/78, rep 40/52, VP1, VP2 and VP3 are disclosed in this U.S. Patent No. 8,692,332. The location and hypervariable regions of AAV8 are also provided. In some embodiments, the vector is an AAV2 variant vector, such as AAV7m8.

The vectors of use in the methods disclosed herein can contain nucleic acid sequences
20 encoding an intact AAV capsid which may be from a single AAV serotype (e.g., AAV2, AAV6, AAV8 or AAV9). As disclosed in U.S. Patent No. 8,692,332, vectors of use can also be recombinant, and thus can contain sequences encoding artificial capsids which contain one or more fragments of the AAV8 capsid fused to heterologous AAV or non-AAV capsid proteins (or fragments thereof). These artificial capsid proteins are selected from non-contiguous portions of
25 the AAV2, AAV6, AAV8 or AAV9 capsid or from capsids of other AAV serotypes. For example, a rAAV vector may have a capsid protein comprising one or more of the AAV8 capsid regions selected from the VP2 and/or VP3, or from VP1, or fragments thereof selected from amino acids 1 to 184, amino acids 199 to 259; amino acids 274 to 446; amino acids 603 to 659; amino acids 670 to 706; amino acids 724 to 738 of the AAV8 capsid, see SEQ ID NO: 2 of U.S. Patent No.
30 8,692,332. In another example, it may be desirable to alter the start codon of the VP3 protein to GTG. Alternatively, the rAAV may contain one or more of the AAV serotype 8 capsid protein hypervariable regions, for example aa 185- 198; aa 260-273; aa447-477; aa495-602; aa660-669; and aa707-723 of the AAV8 capsid set forth in SEQ ID NO: 2 of U.S. Patent No. 8,692,332.

In some embodiments, a recombinant adeno-associated virus (rAAV) is generated having an

AAV serotype 2 capsid. To produce the vector, a host cell which can be cultured that contains a nucleic acid sequence encoding an AAV serotype 2 capsid protein, or fragment thereof, as defined herein; a functional rep gene; a minigene composed of, at a minimum, AAV inverted terminal repeats (ITRs) and a transgene, such as encoding a KLK10 protein, optionally operably linked to a
5 *KLK10* promoter; and sufficient helper functions to permit packaging in the AAV2/9 capsid protein. The components required to be cultured in the host cell to package an AAV minigene in an AAV capsid may be provided to the host cell in trans. Alternatively, any one or more of the required components (e.g., minigene, rep sequences, cap sequences, and/or helper functions) may be provided by a stable host cell which has been engineered to contain one or more of the required
10 components using methods known to those of skill in the art. In some embodiments, a stable host cell will contain the required component(s) under the control of an inducible promoter or a tissue specific promoter. Similar methods can be used to generate a rAAV2, rAAV8 or rAAV9 vector and/or virion.

An endothelial cell specific promoter can be included in the AAV vectors. In some
15 embodiments, the promoter is an ICAM2 promoter, endoglin promoter, or a CDH5 promoter. In other embodiments, the promoter is a synthetic endothelial cell-specific promoter, see Dai et al., J. Virol. 2004 Jun; 78(12): 6209–6221, doi: 10.1128/JVI.78.12.6209-6221.2004, incorporated herein by reference.

In other embodiments, component(s), such as, but not limited to, a transgene encoding a
20 KLK10 protein or the fusion protein, can be under the control of a constitutive promoter. A non-limiting example of a suitable constitutive promoter is the cytomegalovirus promoter. Additional non-limiting examples are the ubiquitin or a chicken β -actin promoter. Promoters of use are also disclosed in the section above. Additional promoters are disclosed above.

In still another alternative, a selected stable host cell may contain selected component(s)
25 under the control of a constitutive promoter and other selected component(s) under the control of one or more inducible promoters, such as for the production of rAAV in a packaging host cell. For example, a stable host cell may be generated which is derived from 293 cells (which contain E1 helper functions under the control of a constitutive promoter), but which contains the rep and/or cap proteins under the control of inducible promoters. Still other stable host cells may be generated by
30 one of skill in the art.

The minigene, rep sequences, cap sequences, and helper functions required for producing a rAAV can be delivered to the packaging host cell in the form of any genetic element which transfer the sequences carried thereon. The selected genetic element may be delivered by any suitable method, including those described herein. The methods used to construct vectors are known to

those with skill in nucleic acid manipulation and include genetic engineering, recombinant engineering, and synthetic techniques. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. Similarly, methods of generating rAAV virions are well known and the selection of a suitable method is not a limitation on the present invention. See, e.g., K. Fisher et al, *J. Virol.*, 70:520-532 (1993) and U.S. Patent No. 5,478,745. In some embodiments, selected AAV components can be readily isolated using techniques available to those of skill in the art from an AAV serotype, including AAV8. Such AAV may be isolated or obtained from academic, commercial, or public sources (e.g., the American Type Culture Collection, Manassas, Va.). Alternatively, the AAV sequences may be obtained through synthetic or other suitable means by reference to published sequences such as are available in the literature or in databases such as, e.g., GENBANK®.

Pharmaceutical Compositions and Methods of Treatment

Atherosclerosis is an inflammatory disease that preferentially occurs in branched or curved arterial regions exposed to disturbed flow (*d-flow*), while areas of stable flow (*s-flow*) are generally protected from atherosclerosis. It is disclosed herein that KLK10 mediates the anti-atherogenic effects of *s-flow*, while the loss of KLK10 under *d-flow* conditions leads to pro-atherogenic effects (see the Examples section). Further, KLK10 is produced under *s-flow* in endothelial cells but is downregulated under *d-flow* conditions, and KLK10 protects against endothelial inflammation, barrier dysfunction, and atherosclerosis. KLK10 inhibits endothelial inflammation in a protease activated receptor-1/2 (PAR1/2)-dependent manner, but without directly cleaving the receptors.

Methods are disclosed herein for treating atherosclerosis and/or decreasing arterial endothelial inflammation in a subject. Methods are also disclosed for reducing monocyte adhesion to blood vessels, inhibiting inflammation in blood vessels, and/or protecting the endothelial permeability barrier in blood vessels in the subject. Methods are also disclosed for treating a subject with atherosclerosis, a stroke, peripheral artery disease, and/or or a myocardial infarction.

The disclosed methods include administering to the subject a therapeutically effective amount of a KLK10 protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein. Suitable KLK10 proteins, fusion proteins, nucleic acid molecules and vectors are disclosed above. These are all of use in these methods.

Administration can be systemic or local. Systemic administration may be by any suitable route, such as, but not limited to, parenteral administration (intravenously, intramuscularly, intraperitoneally, or subcutaneously), or oral administration. In some embodiments, the KLK10

protein, the fusion protein, nucleic acid molecule or vector is administered locally to a blood vessel of the subject. Local administration can include administration to a specific lesion, such as an atherosclerotic plaque. In specific, non-limiting embodiments, the KLK10 protein, the fusion protein, or the nucleic acid molecule is administered to the subject in a stent.

5 In any of the disclosed methods, the subject can be a human or veterinary subject. Veterinary subjects include domesticated animals or household pets, such as dogs, cats, horses, cows, and pigs. Non-human primates and wild animals can also be treated. The subject (such as a human subject) can be any age, such as a child or an adult, for example, a younger adult, middle-aged adult, or older adult. Most typically, a human subject is a middle-aged adult or older adult,
10 and thus is at least 40 years of age, such as at least 45 years of age, such as at least 50, 55, 60, 65, or 70 years of age.

In some embodiments, the method includes selecting a subject for treatment. In certain non-limiting examples, a subject is selected that has atherosclerosis, a stroke, peripheral artery disease, and/or or myocardial infarction. In some embodiments, the subject has been determined to
15 be at risk for cardiovascular disease based on risk factors, such as, but not limited to, Framingham risk factors, or guidelines jointly issued by the American Heart Association and American College of Cardiology. In specific non-limiting examples, the method can include evaluating a subject to determine if the subject is at risk for cardiovascular disease using Framingham risk factors. These risk factors include age, gender, whether the subject smokes, blood pressure, total cholesterol level,
20 and high-density lipoprotein cholesterol level.

The Framingham Risk Score is a gender-specific algorithm used to estimate the 10-year cardiovascular risk of a subject using specific factors. The Framingham Risk Score was first developed based on data obtained from the Framingham Heart Study, to estimate the 10-year risk of developing coronary heart disease (*See*, Third Report of the National Cholesterol Education
25 Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) final report, *Circulation* 2002 Dec 17;106(25):3143-421, incorporated herein by reference). The method can include evaluation of a subject to determine if the subject is at risk for cardiovascular disease using risk factors, such as, but not limited to, Framingham risk factors and/or guidelines jointly issues by the American Heart Association and
30 American College of Cardiology.

Framingham risk factors include age, gender, low density lipoprotein (LDL) cholesterol level, whether the subject smokes, blood pressure (and whether the subject is receiving pharmacological treatment for hypertension), total cholesterol level, and high-density lipoprotein (HDL) cholesterol level. Programs for this evaluation are available on the internet, such as at the

U.S. National Heart, Lung, and Blood Institute (NHLBI) website. The disclosed methods can include (a) selecting a subject for treatment based on the Framingham risk factor and/or (b) evaluating the Framingham risk factors as part of the treatment protocol.

In some embodiments, the disclosed methods are of use to treat a subject with atherosclerosis. The subject can have atherosclerotic heart disease. These subject can e selected for treatment. In some embodiments the subject also be administered a therapeutically effective amount of a statin, niacin, a fibrate, a bile acid binding resin, a cholesterol absorption inhibitor, a PCSK9-targeting drug, an LDL-targeting drug or an HDL-targeting drug.

In some embodiments, the disclosed methods are of use to treat a subject who has a myocardial infarction, or previously had a myocardial infarction. Generally, these subjects have cardiac tissue death caused by ischemia. Acute myocardial infarction (AMI), or a "heart attack," occurs when localized myocardial ischemia causes the development of a defined region of tissue death. AMI is most often caused by rupture of an atherosclerotic lesion in a coronary artery. This causes the formation of a thrombus that plugs the artery, stopping it from supplying blood to the region of the heart that it supplies. These subjects can be selected for treatment.

The disclosed methods are of use to treat a subject that has cardiac ischemia. Severe and prolonged ischemia produces a region of necrosis spanning the entire thickness of the myocardial wall. Such a transmural infarct usually causes ST segment elevation. Less severe and protracted ischemia can arise when coronary occlusion is followed by spontaneous reperfusion; an infarct-related artery is not completely occluded; occlusion is complete, but an existing collateral blood supply prevents complete ischemia; or the oxygen demand in the affected zone of myocardium is smaller. Under these conditions, the necrotic zone may be mainly limited to the subendocardium, typically causing non-ST segment elevation MI. A subject with any of these changes can be selected for treatment.

In other embodiments the disclosed methods are of use to treat a subject that has a vascular disorder, thrombotic stroke, peripheral vascular disease, restenosis, acute coronary syndrome, or reperfusion myocardial injury.

The subject can have a myocardial infarction or cardiac ischemia, and can also be administered a therapeutically effective amount of an antiplatelet agent, an anticoagulation agent, a lipid or blood pressure regulating agent, or an anti-oxidant. Exemplary lipid regulating agents are statin, niacin, PCSK9-targeting drug, bile acid binding resin, or HDL-cholesterol targeting drug.

In any embodiment disclosed herein, the subject can be administered a therapeutically effective amount of an antioxidant, such as N-acetylcysteine, vitamin C, beta carotene, or vitamin E. In a specific non-limiting example, the subject can be administered a therapeutically effective

amount of N-acetylcysteine.

A KLK10 protein, a fusion protein comprising the KLK10 protein, a nucleic acid molecule encoding the KLK protein or the fusion protein, a vector comprising the nucleic acid molecule peptide, or a composition comprising any of the above, may be administered sequentially or simultaneously (such as separately) with the one or more additional therapeutic agents. Simultaneous administration refers to the administration of at least two therapeutics by the same route and at the same time or at substantially the same time. Separate administration refers to administering at least two therapeutics at the same time or at substantially the same time by different routes. Sequential administration refers to administration of at least two therapeutics at different times, the administration route being identical or different. More particularly, sequential administration refers to the whole administration of one of the therapeutics before administration of the other or others commences. It is thus possible to administer one of the therapeutics over several minutes, hours, or days before administering the other therapeutic or therapeutics.

Compositions comprising a KLK10 protein, a fusion protein comprising the KLK10 protein, a nucleic acid molecule encoding the KLK protein or the fusion protein, or a vector comprising the nucleic acid molecule are also disclosed that are of use in the present methods. Without limitation, these compositions can be used to decrease arterial endothelial inflammation, reduce monocyte adhesion to blood vessels, and/or protecting the endothelial permeability barrier in blood vessels. These compositions can be used to treat a subject with vascular disorder, thrombotic stroke, peripheral vascular disease, restenosis, acute coronary syndrome, or reperfusion myocardial injury.

The disclosed compositions can further comprise one or more additional components, such as one or more other therapeutics, such as an antihyperlipidemic and/or anti-inflammatory agent (such as a cholesterol absorption inhibitor and/or a cholesterol lowering agent), and/or one or more pharmaceutically acceptable carriers. In any embodiment disclosed herein, the subject can be administered a therapeutically effective amount of an antioxidant, such as N-acetylcysteine, vitamin C, beta carotene, or vitamin E. In a specific non-limiting example, the subject can be administered a therapeutically effective amount of N-acetylcysteine.

Compositions of the present disclosure are not limited to any particular one or more additional components. In some embodiments, the one or more antihyperlipidemic and/or anti-inflammatory agents includes a statin, a bile acid sequestrant (resin), nicotinic acid, a fibric acid derivative (a fibrate), and/or an HMG-CoA reductase inhibitor. In a specific, non-limiting example, the antihyperlipidemic and/or anti-inflammatory agents includes one or more of

atorvastatin, simvastatin, pravastatin, fluvastatin, lovastatin, pitavastatin, rosuvastatin, clinofibrate, clofibrate, simfibrate, fenofibrate, bezafibrate, colestimide, and colestyramine.

Compositions described herein can be administered to a subject using any of the disclosed methods. Pharmaceutical compositions can be provided as parenteral compositions, such as for
5 injection or infusion. Such compositions are formulated generally by mixing a disclosed therapeutic agent at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, for example one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. The active ingredient, optionally together with excipients, can also
10 be in the form of a lyophilisate and can be made into a solution prior to parenteral administration by the addition of suitable solvents. Solutions such as those that are used, for example, for parenteral administration can also be used as infusion solutions.

Pharmaceutical compositions can include an effective amount of the polypeptide, nucleic acid, or dispersed (for example, dissolved or suspended) in a pharmaceutically acceptable carrier or
15 excipient. Pharmaceutically acceptable carriers and/or pharmaceutically acceptable excipients are known in the art and are described, for example, in *Remington's Pharmaceutical Sciences*, by E. W. Martin, Mack Publishing Co., Easton, PA, 19th Edition (1995).

The nature of the carrier will depend on the particular mode of administration being employed. For example, parenteral formulations usually contain injectable fluids that include
20 pharmaceutically and physiologically acceptable fluids such as water, physiological saline, balanced salt solutions, aqueous dextrose, glycerol or the like as a vehicle. For solid compositions (such as powder, pill, tablet, or capsule forms), conventional non-toxic solid carriers can include, for example, pharmaceutical grades of mannitol, lactose, starch or magnesium stearate. In addition, pharmaceutical compositions to be administered can contain minor amounts of non-toxic auxiliary
25 substances, such as wetting or emulsifying agents, preservatives, and pH buffering agents and the like, for example sodium acetate or sorbitan monolaurate.

The disclosed composition can include solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any
30 conventional media or agent is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions. For example, certain pharmaceutical compositions can include the vectors or viruses in water, mixed with a suitable surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and

in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

Administration may be provided as a single administration, a periodic bolus (for example, into a vessel, or as continuous infusion from an internal reservoir (for example, from an implant disposed (see, U.S. Pat. Nos. 5,443,505 and 5,766,242)) or from an external reservoir (for example, from an intravenous bag). Administration of a therapeutic agent can be performed once, or can be performed repeatedly, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 times. Administration can be performed biweekly, weekly, every other week, monthly, or every 2, 3, 4, 5, or 6 months.

Individual doses are typically not less than an amount required to produce a measurable effect on the subject and may be determined based on the pharmacokinetics and pharmacology of the subject composition or its by-products, and thus based on the disposition of the composition within the subject. This includes consideration of the route of administration as well as dosage amount, which can be adjusted for specific applications, such as intravenous administration. Effective amounts of dose and/or dose regimen can readily be determined empirically from preclinical assays, from safety and escalation and dose range trials, individual clinician-patient relationships, as well as *in vitro* and *in vivo* assays.

Nucleic acid molecules can be delivered by microinjection, electroporation, lipid-mediated transfection, peptide-mediated delivery, nanoparticle mediated deliver, dendrimer mediated delivery, or other methods known in the art. An appropriate dose depends on the subject being treated (e.g., human or nonhuman primate or other mammal), age and general condition of the subject to be treated, the severity of the condition being treated, the mode of administration of the vector/virion, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. Thus, a "therapeutically effective amount" will fall in a relatively broad range that can be determined through clinical trials.

Components can be administered by continuous release for a particular period from a sustained release drug delivery device. A therapeutic agent can be incorporated, for example, into a stent.

In some embodiments, for *in vivo* injection when a viral vector is used, i.e., injection of a viral vector encoding KLK10 or a fusion protein thereof directly to the subject, a therapeutically effective dose will be on the order of from about 10^5 to 10^{16} of the AAV virions, such as 10^8 to 10^{14} AAV virions. The dose, of course, depends on the efficiency of transduction, promoter strength, the stability of the message and the protein encoded thereby, and clinical factors. Effective dosages can be readily established through routine trials establishing dose response curves.

In some embodiments, if the nucleic acid molecule is included in an AAV vector, an

effective amount to achieve a change will be about 1×10^8 vector genomes or more, in some cases about 1×10^9 , about 1×10^{10} , about 1×10^{11} , about 1×10^{12} , or about 1×10^{13} vector genomes or more, in certain instances, about 1×10^{14} vector genomes or more, and usually no more than about 1×10^{15} vector genomes. In some embodiments, the amount of vector that is delivered is about 1×10^{14} vectors or less, for example about 1×10^{13} , about 1×10^{12} , about 1×10^{11} , about 1×10^{10} , or about 1×10^9 vectors or less, in certain instances about 1×10^8 vectors, and typically no less than 1×10^8 vectors. In some non-limiting examples, the amount of vector genomes that is delivered is about 1×10^{10} to about 1×10^{11} vectors. In additional non-limiting examples, the amount of vector that is delivered is about 1×10^{10} to about 1×10^{12} vector genomes.

In some embodiments, the amount of pharmaceutical composition to be administered may be measured using plaque forming units (pfu). In some embodiments, pfu refers to the ratio, or multiple of vector or viral genomes to the cells to which the nucleic may be delivered. In some embodiments, the pfu may be about 1×10^6 . In some cases, the pfu can be about 1×10^5 to about 1×10^7 . In some cases, the pfu may be about 1×10^4 to about 1×10^8 . In some cases, recombinant viruses of the disclosure are at least about 1×10^1 , about 1×10^2 , about 1×10^3 , about 1×10^4 , about 1×10^5 , about 1×10^6 , about 1×10^7 , about 1×10^8 , about 1×10^9 , about 1×10^{10} , about 1×10^{11} , about 1×10^{12} , about 1×10^{13} , about 1×10^{14} , about 1×10^{15} , about 1×10^{16} , about 1×10^{17} , and about 1×10^{18} pfu. In some cases, recombinant viruses of this disclosure are about 1×10^8 to 1×10^{14} pfu.

In some the amount of pharmaceutical composition delivered comprises about 1×10^8 to about 1×10^{15} particles of recombinant viruses, about 1×10^9 to about 1×10^{14} particles of recombinant viruses, about 1×10^{10} to about 1×10^{13} particles of recombinant viruses, or about 1×10^{11} to about 1×10^{12} particles of recombinant viruses (see U.S. Published Patent Application No. 2015/0259395, incorporated herein by reference).

Dosage treatment may be a single dose schedule or a multiple dose schedule to ultimately deliver the amount specified above. Moreover, the subject may be administered as many doses as appropriate. Thus, the subject may be given, e.g., 10^5 to 10^{16} AAV virions in a single dose, or two, four, five, six or more doses that collectively result in delivery of, e.g., 10^5 to 10^{16} AAV virions. One of skill in the art can readily determine an appropriate number of doses to administer.

In some embodiments, an AAV is administered at a dose of about 1×10^{11} to about 1×10^{14} viral particles (vp)/kg. In some examples, the AAV is administered at a dose of about 1×10^{12} to about 8×10^{13} vp/kg. In other examples, the AAV is administered at a dose of about 1×10^{13} to about 6×10^{13} vp/kg. In specific non-limiting examples, the AAV is administered at a dose of at least about 1×10^{11} , at least about 5×10^{11} , at least about 1×10^{12} , at least about 5×10^{12} , at least

about 1×10^{13} , at least about 5×10^{13} , or at least about 1×10^{14} vp/kg. In other non-limiting examples, the rAAV is administered at a dose of no more than about 5×10^{11} , no more than about 1×10^{12} , no more than about 5×10^{12} , no more than about 1×10^{13} , no more than about 5×10^{13} , or no more than about 1×10^{14} vp/kg. In one non-limiting example, the AAV is administered at a dose of about 1×10^{12} vp/kg. The AAV can be administered in a single dose, or in multiple doses (such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 doses) as needed for the desired therapeutic results.

The pharmaceutical compositions can contain the vector, such as the AAV vector, and/or virions and a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the composition and that may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, glycerol, and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts, such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like, and salts of organic acids, such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

In some embodiments, the excipients confer a protective effect on the AAV virion such that loss of AAV virions as well as transduceability resulting from formulation procedures, packaging, storage, transport, and the like is minimized. Therefore, these excipient compositions are considered "virion-stabilizing" because they provide higher AAV virion titers and higher transduceability levels than their non-protected counterparts, as measured using standard assays (see, for example, Published U.S. Application No. 2012/0219528, incorporated herein by reference). Therefore, these compositions demonstrate "enhanced transduceability levels" compared with compositions lacking the particular excipients described herein and are, thus, more stable than their non-protected counterparts.

Exemplary excipients that can be used to protect the AAV virion from activity degradative conditions include, but are not limited to, detergents, proteins (e.g., ovalbumin and bovine serum albumin), amino acids (e.g., glycine), polyhydric and dihydric alcohols (e.g., polyethylene glycols (PEG) of varying molecular weights, such as PEG-200, PEG-400, PEG-600, PEG-1000, PEG-1450, PEG-3350, PEG-6000, PEG-8000 and any molecular weights in between these values, with molecular weights of 1500 to 6000 preferred), propylene glycols (PG), and sugar alcohols (such as a carbohydrate, preferably, sorbitol). The detergent, when present, can be an anionic, a cationic, a zwitterionic, or a nonionic detergent. An exemplary detergent is a nonionic detergent. One

suitable type of nonionic detergent is a sorbitan ester (e.g., polyoxyethylenesorbitan monolaurate (TWEEN®-20) polyoxyethylenesorbitan monopalmitate (TWEEN®-40), polyoxyethylenesorbitan monostearate (TWEEN®-60), polyoxyethylenesorbitan tristearate (TWEEN®-65), polyoxyethylenesorbitan monooleate (TWEEN®-80), polyoxyethylenesorbitan trioleate (TWEEN®-85), such as TWEEN®-20 and/or TWEEN®-80). These excipients are commercially available from a number of vendors, such as Sigma, St. Louis, Mo.

The amount of the various excipients present in any of the disclosed compositions, including AAV, varies and is readily determined by one of skill in the art. For example, a protein excipient, such as BSA, if present, can be present at a concentration of between 1.0 weight (wt.) % to about 20 wt. %, preferably 10 wt. %. If an amino acid, such as glycine, is used in the formulations, it can be present at a concentration of about 1 wt. % to about 5 wt. %. A carbohydrate, such as sorbitol, if present, can be present at a concentration of about 0.1 wt % to about 10 wt. %, such as between about 0.5 wt. % to about 15 wt. % or about 1 wt. % to about 5 wt. %. If polyethylene glycol is present, it can generally be present on the order of about 2 wt. % to about 40 wt. %, such as about 10 wt. % to about 25 wt. %. If propylene glycol is used in the subject formulations, it will typically be present at a concentration of about 2 wt. % to about 60 wt. %, such as about 5 wt. % to about 30 wt. %. If a detergent, such as a sorbitan ester (TWEEN®), is present, it can be present at a concentration of about 0.05 wt. % to about 5 wt. %, such as between about 0.1 wt. % and about 1 wt % (see U.S. Published Patent Application No. 2012/0219528, which is incorporated herein by reference). In one example, an aqueous virion-stabilizing formulation comprises a carbohydrate, such as sorbitol, at a concentration of between 0.1 wt. % to about 10 wt. %, such as between about 1 wt. % to about 5 wt. %, and a detergent, such as a sorbitan ester (TWEEN®) at a concentration of between about 0.05 wt. % and about 5 wt. %, such as between about 0.1 wt. % and about 1 wt. %. Virions are generally present in the composition in an amount sufficient to provide a therapeutic effect when given in one or more doses, as defined above.

Kits

Kits are also provided. The kit can include a KLK10 protein, a fusion protein including the KLK10 protein, a nucleic acid molecule encoding the KLK10 protein, a vector including the nucleic acid molecule, or a therapeutic form, such as a stent, including one of these therapeutic agents. The kit can further include buffers or other therapeutic agents.

The kit can include a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, etc. The containers

may be formed from a variety of materials such as glass or plastic. The container typically holds a composition including one or more of the probes, primers and/or antibodies. In several embodiments the container may have a sterile access port.

A label or package insert indicates that the therapeutic agent is of use of the treatment of a subject. The label or package insert typically will further include instructions for use, such as particular dilutions and dosages. The package insert typically includes instructions customarily included in commercial packages of products that contain information about the indications, usage, contraindications and/or warnings concerning the use of such products. The instructional materials may be written, in an electronic form (such as a computer diskette or compact disk) or may be visual (such as video files). The kits may also include additional components to facilitate the particular application for which the kit is designed. The kits may additionally include buffers and other reagents routinely used for dilution of a therapeutic agent. Such kits and appropriate contents are well known to those of skill in the art.

EXAMPLES

A partial carotid ligation (PCL) mouse model of atherosclerosis and transcriptomic studies were used to identify hundreds of flow-sensitive genes in endothelial cells (ECs) that change based on *d-flow* in the left carotid artery (LCA) as compared to the *s-flow* in the right carotid artery (RCA) (Nam D, et al. *Am. J. Physiol-Heart C.* 2009, 297:H1535-H1543; Ni C-W, et al. *Blood.* 2010, 116:e66-e73). Flow-sensitive genes include Krüppel-like Factor 2 (KLF2) (Dekker RJ, et al. *Blood.* 2002, 100:1689-1698), Krüppel-like Factor 4 (KLF4) (Sangwung P, et al. *JCI Insight.* 2017, 2:e91700-e91700), bone morphogenetic protein 4 (Jo H, et al. *Antioxid. Redox Signal.* 2006, 8:1609-1619), hypoxia inducible factor-1a pathway regulating gene UBE2c (Feng S, et al. *Arterioscler. Thromb. Vasc. Biol.* 2017, 37:2087-2101; Esmerats JF, et al. *Arterioscler. Thromb. Vasc. Biol.* 2019, 39:467-481), sterol regulatory element binding protein 2 (Xiao H, et al. *Circulation.* 2013, 128:632-642), PPAP2B (Wu C, et al. *Circ. Res.* 2015, 117:e41-e53), ZBTB46 (Wang Y, et al. *Lab. Invest.* 2019, 99:305-318), JCAD/KIAA1462 (Xu S, et al. *Eur. Heart J.* 2019, 40:2398-2408), JMJD2b (Glaser SF, et al. *Proc. Natl. Acad. Sci. U.S.A.* 2020, 117:4180-4187), endothelial nitric oxide synthase (Boo YC, et al. *Am. J. Physiol-Heart C.* 2002, 283:H1819-H1828), and several flow-sensitive microRNAs (Son DJ, et al. *Nature Communications.* 2013, 4:3000). Among the flow-sensitive genes, Kallikrein related-peptidase 10 (*KLK10*) was identified as one of the most flow-sensitive, with high expression under *s-flow* and low expression under *d-flow* conditions (Ni C-W, et al. *Blood.* 2010, 116:e66-e73).

The effect of KLK10 on the endothelial inflammatory response was assessed as measured

by monocyte adhesion under flow-conditions *in vitro* and *in vivo*. The results demonstrate that either KLK10 overexpression using plasmids or rKLK10 treatment protects against EC inflammation both *in vitro* and *in vivo* under TNF α or *d-flow* conditions, whereas KLK10 downregulation under *d-flow* conditions or KLK10 knockdown using siRNA induces inflammation.

5 KLK10 treatment also reduced the permeability barriers of ECs, demonstrating the protective role of KLK10 in endothelial inflammation and barrier function. It was also demonstrated that treatment with KLK10 by either rKLK10 or a KLK10 expression vector inhibited atherosclerosis development in *ApoE*^{-/-} mice. Furthermore, in human samples, it was demonstrated that KLK10 expression was predominantly present in non-diseased arteries, but was significantly reduced in

10 arteries with plaques.

Example 1

Materials and Methods

Mouse studies: All animal studies were performed with male C57BL/6 or *ApoE*^{-/-} mice (Jackson Laboratory), were approved by the Institutional Animal Care and Use Committee of Emory University, and were performed in accordance with the established guidelines and regulations consistent with federal assurance. All studies using mice were carried out with male mice at 6-10 weeks to reduce the sex-dependent variables. For partial carotid ligation studies, mice at 10 weeks were anesthetized and 3 of 4 caudal branches of the LCA (left external carotid, internal carotid, and occipital artery) were ligated with 6-0 silk suture, but the superior thyroid artery was left intact. Development of *d-flow* with characteristic low and oscillating shear stress in each mouse was determined by ultrasound measurements as previously described (Nam D, et al. *Am. J. Physiol-Heart C.* 2009, 297:H1535-H1543). Following the partial ligation, mice were either fed chow-diet for 2 days or high-fat diet for atherosclerosis studies for 3 weeks as specified in each

25 study.

Endothelial-enriched RNA was prepared from the LCA and the contralateral RCA control 48 h after the partial ligation as described previously (Nam D, et al. *Am. J. Physiol-Heart C.* 2009, 297:H1535-H1543). For *en face* immunostaining, mice were euthanized under CO₂ and the aortas were pressure-fixed with 10% formalin saline (Nam D, et al. *Am. J. Physiol-Heart C.* 2009, 297:H1535-H1543). The aortas were carefully cleaned *in situ*, and the aortic arches and thoracic aortas were dissected, fixed in ice-cold acetone for 5 min, permeabilized using 0.1% Triton-X100 in PBS for 15 min, blocked for 2 h with 10% donkey serum, and incubated with anti-KLK10 (BiossUSA bs-2531R, 1:100) and anti-VCAM1 (Abcam ab134047, 1:100) primary antibodies overnight at 4°C followed by Alexa Fluor-647 secondary antibodies (ThermoFisher Scientific,

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1:500) for 2 h at room temperature. Aortas were opened and the lesser curvatures (LC) and greater curvatures (GC) of each arch were separated. Aortas were then mounted on glass slides with VectaShield that contained DAPI (Vector Laboratories). *En face* images were collected as a Z-stack with a Zeiss LSM 800 confocal microscope. For mouse frozen section staining studies, fresh mouse aortas were placed in Tissue-Tek OCT compound, snap-frozen in liquid nitrogen, and sectioned at 7 μ m as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000).

TABLE 1. qPCR Primers (SEQ ID NOs: 15-24)

Primer (custom)	Sequence
h_KLK10 For	GAGTGTGAGGTCTTCTACCCTG
h_KLK10 Rev	ATGCCTTGGAGGGTCTCGTCAC
m_KLK10 For	CGC TAC TGA TGG TGC AAC TCT
m_KLK10 Rev	ATA GTC ACG CTC GCA CTG G
H/M 18S For	AGGAATTGACGGAAGGGCACCA
H/M 18S Rev	GTGCAGCCCCGGACATCTAAG
h_VCAM1 For	GATTCTGTGCCCACAGTAAGGC
h_VCAM1 Rev	TGGTCACAGAGCCACCTTCTTG
h_ICAM1 For	AGCGGCTGACGTGTGCAGTAAT
h_ICAM1 Rev	TCTGAGACCTCTGGCTTCGTCA

Immunohistochemical staining of sections from human coronaries: For human coronaries arteries, 2mm cross sections of the left anterior descending arteries were obtained from de-identified human hearts not suitable for cardiac transplantation donated to LifeLink of Georgia. The de-identified donor information is shown in Table 2. Tissues were fixed in 10% neutral buffered formalin overnight, embedded in paraffin, and 7 μ m sections were taken, and stained as previously described (Chang K, et al. *Circulation*. 2007, 116:1258-1266; Kim CW, et al. *Arterioscler. Thromb. Vasc. Biol.* 2013, 33:1350-1359). Sections were deparaffinized and antigen retrieval was performed as described previously (Chang K, et al. *Circulation*. 2007, 116:1258-1266; Kim CW, et al. *Arterioscler. Thromb. Vasc. Biol.* 2013, 33:1350-1359). Sections were then permeabilized using 0.1% Triton-X100 in PBS for 15 minutes, blocked for 2h with 10% goat serum, and incubated with anti-KLK10 (BiossUSA bs-2531R, 1:100) primary antibody overnight at 4°C followed by Alexa Fluor-647 (ThermoFisher Scientific, 1:500) secondary antibody for 2 h at room temperature (Table 1). Nuclei were counter-stained with DAPI (Vector Laboratories,

Burlingame, Calif). Hematoxylin and Eosin staining (American Mastertech) and plaque area quantification using ImageJ software (NIH) were done as previously described (Chang K, et al. *Circulation*. 2007, 116:1258-1266; Kim CW, et al. *Arterioscler. Thromb. Vasc. Biol.* 2013, 33:1350-1359). All confocal images were taken with a Zeiss (Jena, Germany) LSM800 confocal microscope.

TABLE 2. Patient Characteristics

	Age (Mean $\pm$ SEM)	Stroke	Hypertension	Diabetes
Total (n=10)	47.9 $\pm$ 3.07	5	7	2
Sex				
Male (n=8)	48.37 $\pm$ 3.29	4	5	1
Female (n=2)	46 $\pm$ 6	1	2	1
Race				
White (n=6)	50 $\pm$ 4.77	3	4	0
Black (n=3)	46.33 $\pm$ 3.48	2	3	2
Hispanic (n=1)	40	0	0	0

Cell culture and in vitro shear stress study: HAECs were obtained from Lonza and maintained in EGM2 medium (Lonza) supplemented with 10% fetal bovine serum (Hyclone), 1% bovine brain extract, 10mM L-glutamine, 1 μ g/mL hydrocortisone hemisuccinate, 50 μ g/mL ascorbic acid, 5 ng/mL EGF, 5 ng/mL VEGF, 5 ng/mL FGF, and 15 ng/mL IGF-1 as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000). HUVECs were purchased from BD Biosciences, cultured in M199 media (Cellgro) supplemented with 20% fetal bovine serum (Hyclone), 1% bovine brain extract, 10mM L-glutamine, and 0.75 U/mL heparin sulfate as previously described (Ni C-W, et al. *Am. J. Physiol-Heart C*. 2011, 300:H1762-H1769). All ECs were grown at 5% CO₂ and 37°C and used between passages 5 and 9. THP-1 monocytes were obtained from ATCC and maintained in RPMI-1640 medium supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol at 5% CO₂ and 37°C as previously described (Ni C-W, et al. *Am. J. Physiol-Heart C*. 2011, 300:H1762-H1769). For flow experiments, confluent HAECs or HUVECs were exposed to steady unidirectional laminar shear stress (LS, 15 dyn/cm²) or bidirectional oscillatory shear stress (OS, $\pm$ 5 dyn/cm² at 1 Hz), mimicking *s-flow* and *d-flow* conditions, respectively, using the cone-and-plate viscometer for 24h experiments, as previously reported (Jo H, et al. *Antioxid. Redox Signal*. 2006, 8:1609-1619; Chang K, et al. *Circulation*. 2007, 116:1258-1266).

rKLK10 and KLK10 Plasmids: Initially, human rKLK10 (Ala34-Asn276 with a 6x N-terminal His tag) produced in *E. coli* (Ray Biotech, 230-00040-10) were used. Additional studies

using human rKLK10 produced in the mammalian CHO-K1 cells validated the initial results. Most studies were carried out using human rKLK10 produced in CHO-K1 cells using a full-length expression vector (pcDNA 3.4, Met1-Asn276). rKLK10 with a 6X C-terminal His tag was affinity purified using HisPur Ni-NTA Resin (Thermo Scientific) per the manufacturer's instruction using the conditioned medium (FIG. 16). Amino acid sequencing analysis of the purified rKLK10 by mass spectrometry showed that the rKLK10 preparation was a mature form expressing Ala34-Asn276.

Overexpression or knockdown experiments in vitro: Cells were transiently transfected with a human KLK10-encoding plasmid (pCMV6-KLK10-Myc-DDK; Origene RC201139) at 0.1-1 µg/mL or as a control a GFP plasmid (PmaxGFP, Lonza, Cat. No. D-00059) using Lipofectamine 3000 (Invitrogen, Cat. No. L3000008) as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000). Alternatively, cells were transfected with KLK10 siRNA (25nM; Dharmacon; J-005907-08), PAR1 (50nM; Dharmacon; L-005094-00-0005), PAR2 (50nM; Dharmacon; L005095-00-0005), or control non-targeting siRNA (25 or 50nM; Dharmacon; Cat. No. D-001810-10-20) using Oligofectamine (Invitrogen, Cat. No. 12252011) as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000).

Quantitative Real-Time Polymerase Chain Reaction (qPCR): Total RNAs were isolated using RNeasy Mini Kit (Qiagen 74106) and reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814). qPCR was performed for genes of interests using VeriQuest Fast SYBR QPCR Master Mix (Affymetrix 75690) with custom designed primers (Table 1) using 18S as house-keeping control as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000).

KLK10 ELISAs: KLK10 secreted into the conditioned cell culture media from HAECs exposed to shear stress was measured by using a human KLK10 ELISA kit (MyBioSource, MBS009286).

Endothelial functional assays: Endothelial migration was measured by the endothelial scratch assay, as previously described (Tressel SL, et al. *Arterioscler. Thromb. Vasc. Biol.* 2007, 27:2150-2156). Briefly, HUVECs were treated with rKLK10 at increasing doses overnight and cell monolayers were scratched with a 200-µL pipette tip. The monolayer was washed once, and the medium was replaced with 2% serum media. After 6h, the number of cells migrated into the scratch area were quantified microscopically using NIH ImageJ.

Endothelial apoptosis was determined using the TUNEL apoptosis assay, as previously described (Alberts-Grill N, et al. *Arterioscler. Thromb. Vasc. Biol.* 2012, 32:623-632). Briefly, HUVECs were treated with rKLK10 at increasing doses overnight and the cells were fixed using

4% PFA for 15 minutes and permeabilized with 0.1% Triton X-100 for 15 minutes. TUNEL staining was then performed using a commercially available kit (Roche, 12156792910) and the number of TUNEL-positive cells were counted using NIH ImageJ.

Endothelial proliferation was determined using Ki67 immunohistochemistry, as previously described (Wang Y, et al. *Lab. Invest.* 2019, 99:305-318). Briefly, HUVECs were treated with rKLK10 at increasing doses overnight and the cells were washed twice with PBS, fixed using 4% PFA for 15 minutes, and permeabilized with 0.1% Triton X-100 for 15 minutes. After blocking with 10% Goat Serum for 2h at RT, cells were incubated overnight at 4°C with rabbit anti-Ki67 primary antibody (Abcam ab15580, 1:100, Table 1). The following day, cells were washed three times with PBS, incubated for 2h at RT protected from light with Alexa-fluor 647-labeled goat anti rabbit IgG (1:500 dilution), and counterstained with DAPI. The number of Ki67 positive cells were counted using NIH ImageJ.

Endothelial tube formation was measured using a Matrigel tube formation assay, as previously described (Tressel SL, et al. *Arterioscler. Thromb. Vasc. Biol.* 2007, 27:2150-2156). Briefly, HUVECs were seeded in a growth factor reduced Matrigel (BD Bioscience) coated 96-well plate and incubated with rKLK10 (100 ng/mL) for 6h at 37°C. Tubule formation was quantified microscopically by measuring tubule length using NIH ImageJ.

Endothelial permeability was determined by FITC-avidin binding to biotinylated gel, as previously described (Dubrovskiy O, et al. *Lab. Invest.* 2013, 93:254-263). Briefly, HAECs were seeded on biotinylated-gelatin and treated with rKLK10 overnight followed by thrombin (5 U/mL) for 4h or OS for 24h as described above. Following the completion of the experiments, FITC-avidin was added to the cells and fluorescent intensity was measured using NIH ImageJ.

Monocyte adhesion to ECs was determined using THP-1 monocytes (ATCC TIB-202) as previously described (Son DJ, et al. *Nature Communications.* 2013, 4:3000). In brief, THP-1 cells (1.5×10^5 cells/mL) were labeled with a fluorescent dye 2',7'-bis(carboxyethyl)-5 (6)-carboxyfluorescein-AM (Thermo Fisher Scientific B1150; 1 mg/mL) in serum-free RPMI medium (Thermo Fisher Scientific 11875093) for 45 minutes at 37°C. After exposure to flow or other experimental treatments, the ECs were washed in RPMI medium before adding 2',7'-bis(carboxyethyl)-5 (6)-carboxyfluorescein-AM-loaded THP-1 cells. After a 30-minute incubation at 37°C under no-flow conditions, unbound monocytes were removed by washing the endothelial dishes 5× with HBSS and cells with bound monocytes were fixed with 4% paraformaldehyde for 10 minutes. Bound monocytes were quantified by counting the number of labeled cells at the endothelium under a fluorescent microscope.

Preparation of whole-cell lysate and immunoblotting: After treatment, cells were washed

3× with ice-cold HBSS and lysed with RIPA buffer containing protease inhibitors (Boston Bioproducts BP-421) (Son DJ, et al. *Nature Communications*. 2013, 4:3000). The protein content of each sample was determined by Pierce BCA protein assay. Aliquots of cell lysate were resolved on 10% to 12% SDS-polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with the following primary antibodies: anti-KLK10 (BiossUSA bs-2531R, 1:1000) anti-GAPDH (Abcam ab23565, 1:2000), anti-β-actin (Sigma-Aldrich A5316, 1:2000), anti-VCAM1 (Abcam ab134047, 1:1000), anti-ICAM1 (Abcam ab53013, 1:1000), and anti-phospho-NFκB (Cell Signaling #3033, 1:1000) overnight at 4°C in 5% milk in TBST at the concentration recommended by the manufacturer, followed by secondary antibody addition for 1h at RT in 5% milk in TBST (Table 1). Protein expression was detected by a chemiluminescence method (Son DJ, et al. *Nature Communications*. 2013, 4:3000).

PAR cleavage assays: Synthetic peptides corresponding to the extracellular domain of PAR1 (AA Ala22-Thr102) and PAR2 (AA Ile26-Thr75) were assembled by automated Fmoc/tBu-solid-phase synthesis (model CS336X; CSBio) followed by cleavage in trifluoroacetic acid (TFA)/phenol/thioanisole/ethanedithiol/water (10:0.75:0.5:0.25:0.5, w/w; 25°C, 90 min) and precipitation with diethyl ether. The crude peptides were purified by reversed-phase high-pressure liquid chromatography and were obtained in the form of their TFA salts. Their masses as well as masses of their theoretical Lys-C protease (Promega) fragments, obtained upon digestion, were confirmed by electrospray-ionization mass spectrometry (maXis ESI-TOF; Bruker). PAR1 and PAR2 peptides (100μM) were then incubated with rKLK10 (100 ng/mL), thrombin (5 U/mL), or trypsin (5 U/mL) for 30 min at 37°C and analyzed by Tricine-SDS-PAGE followed by Coomassie stain (Schägger H. *Nat. Protoc.* 2006, 1:16-22).

PAR1/2-Alkaline Phosphatase (AP) constructs made as previously described (Rana S, et al. *J. Cell Biochem.* 2012, 113:977-984; Mosnier LO, et al. *Blood*. 2012, 120:5237-5246; Bae JS, et al. *J. Thromb. Haemost.* 2008, 6:954-961) were transfected into HAECs (1 μg/mL) for 24 h using Lipofectamine 3000. Cells were treated with rKLK10 (100 ng/mL), thrombin (5 U/mL), or trypsin (5 U/mL) for 30 min. Conditioned media were then collected and analyzed for secreted alkaline phosphatase activity (SEAP) using a commercial kit (T1015, Invitrogen) and a microplate reader (Rana S, et al. *J. Cell Biochem.* 2012, 113:977-984; Mosnier LO, et al. *Blood*. 2012, 120:5237-5246; Bae JS, et al. *J. Thromb. Haemost.* 2008, 6:954-961).

rKLK10 treatment and KLK10 overexpression in C57BL/6 and ApoE^{-/-} mice: Two independent methods were used, rKLK10 and KLK10 plasmid, to treat mice with KLK10. Treatment with rKLK10 was first performed in C57BL/6 mice by administering rKLK10 (0.006-

0.6mg/kg) by tail-vein once. Treatment with rKLK10 was first performed in C57BL/6 mice by administering rKLK10 (0.006-0.6 mg/kg) by tail-vein once. Three days later, mice were euthanized by CO₂ inhalation and *en face* preparation of the aorta was performed as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000). Alternatively, *ApoE*^{-/-} mice on a high-fat diet containing 1.25% cholesterol, 15% fat and 0.5% cholic acid were given the partial carotid ligation surgery and rKLK10 or vehicle was administered by tail-vein once every three days for two weeks as previously described (Son DJ, et al. *Nature Communications*. 2013, 4:3000). Following the completion of the study, mice were euthanized by CO₂ inhalation and the aortas were excised, imaged, and sectioned for IHC (Son DJ, et al. *Nature Communications*. 2013, 4:3000).

KLK10 plasmid overexpression was performed using ultrasound-mediated sonoporation method of gene therapy as reported (Liu R, et al. *ACS Nano*. 2019, 13:5124-5132; Borden MA, et al. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control*. 2005, 52:1992-2002; Shapiro G, et al. *J Control Release*. 2016, 223:157-164). Briefly, perfluoropropane microbubbles encapsulated by DSPC and DSPE-PEG2000 (9:1 molar ratio) were made using the shaking method as previously described (Liu R, et al. *ACS Nano*. 2019, 13:5124-5132; Borden MA, et al. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control*. 2005, 52:1992-2002; Shapiro G, et al. *J Control Release*. 2016, 223:157-164). KLK10 plasmid expressing secreted KLK10 and luciferase (pCMV-Igκ-KLK10-T2A-Luc) from GENEWIZ or luciferase plasmid (pCMV-Luc) from Invitrogen (50μg each) was then mixed with the microbubbles (5×10⁵) and saline to reach 20 μl total volume. Following partial carotid ligation, *ApoE*^{-/-} mice were intramuscular injected to the hind-limbs with the plasmid-microbubble solution. The injected areas of the hind-legs were then exposed to ultrasound (0.35 W/cm²) for 1 min, and repeated 10 days later. At the completion of the study 3 weeks after the partial ligation and on high-fat diet, mice were anesthetized, administered with luciferin (IP; 3.75mg) and imaged for bioluminescence on a Bruker *In Vivo* Xtreme X-ray Imaging System. Mice were then euthanized by CO₂ inhalation and the aortas were excised, imaged, and sectioned for staining as described above.

Serum lipid analysis: Serum lipid analysis was performed at the Cardiovascular Specialty Laboratories (Atlanta, GA) using a Beckman CX7 biochemical analyzer for total cholesterol, triglycerides, HDL and LDL as previously reported (Son DJ, et al. *Nature Communications*. 2013, 4:3000).

Statistical analyses: Statistical analyses were performed using GraphPad Prism software. All of the n numbers represent biological replicates. Error bars depict the standard error of means (SEM). Initially, the data sets were analyzed for normality using the Shapiro-Wilk test (*P*<0.05) and equal variance using the *F* test (*P*>0.05). Data that followed a normal distribution and

possessed equal variance were analyzed using 2-tailed Student *t* test or 1-way ANOVA, where appropriate with Bonferroni post hoc test as needed. In the case where the data showed unequal variances, an unpaired *t* test with Welch correction was performed or Brown-Forsythe and Welch ANOVA for multiple comparisons. If the data failed the Shapiro-Wilk test ($P > 0.05$), a nonparametric Mann-Whitney *U* test was conducted for pairwise comparisons or the Kruskal-Wallis for multiple groups was performed.

Example 2

KLK10 expression in ECs is increased by *s-flow* and decreased by *d-flow* *in vivo* and *in vitro*

Previous mouse gene array data was first validated at the mRNA and protein levels by additional qPCR, immunostaining, Western blots, and ELISA in ECs *in vivo* and *in vitro*. To validate the flow-dependent regulation of KLK10 expression *in vivo*, mouse PCL surgery was performed to induce *d-flow* in the left carotid artery (LCA) while maintaining *s-flow* in right carotid artery (RCA) (FIGS. 1A-1d). Consistent with previous data (Nam D, et al. *Am. J. Physiol-Heart C.* 2009, 297:H1535-H1543; Ni C-W, et al. *Blood.* 2010, 116:e66-e73), KLK10 protein and mRNA expression was significantly higher in ECs in the *s-flow* RCA compared to the *d-flow* LCA (FIGS. 1B-1D). Furthermore, KLK10 expression was observed only in the RCA endothelial layer but not in the medial layer, suggesting that KLK10 is expressed in ECs in the carotid artery region exposed to *s-flow*. In addition, KLK10 protein expression was reduced in the lesser curvature (LC; the athero-prone aortic arch region that is naturally and chronically exposed to *d-flow*) compared to the greater curvature region (GC; the athero-protected aortic arch region that is naturally and chronically exposed to *s-flow*) as shown by *en face* immunostaining (FIGS. 1E-1F).

Next, it was tested whether flow can regulate KLK10 expression *in vitro* using human aortic ECs (HAECs) exposed to unidirectional laminar shear (LS at 15 dynes/cm²) or oscillatory shear (OS at ± 5 dynes/cm²) for 24 h using the cone and plate viscometer, mimicking *s-flow* and *d-flow* conditions *in vivo*, respectively (Jo H, et al. *Antioxid. Redox Signal.* 2006, 8:1609-1619; Chang K, et al. *Circulation.* 2007, 116:1258-1266). KLK10 mRNA (FIG. 1G), protein in cell lysates (FIGS. 1H-1I), and secreted protein in the conditioned media were decreased by OS and increased by LS (FIG. 1J), confirming the role of KLK10 as a flow-sensitive gene and protein *in vivo* and *in vitro*.

Example 3

KLK10 regulates the endothelial inflammation and permeability

KLK10 regulation of EC function was assessed by evaluating the role of KLK10 in endothelial inflammatory response, tube formation, migration, proliferation, and apoptosis, each of

which play critical roles in the pathogenesis of atherosclerosis. Treatment of human umbilical vein ECs (HUVECs) with rKLK10 significantly inhibited migration and tube formation, but not proliferation and apoptosis (FIG. 9). Given the importance of endothelial inflammation in atherosclerosis, the role of KLK10 in ECs exposed to TNF α or shear stress was further tested.

5 Treatment of HAECs with plasmids to overexpress KLK10 reduced THP1 monocyte adhesion to the ECs in response to TNF α and under basal conditions (FIG. 2A; FIG. 10). Next, HAECs were pretreated overnight with increasing concentrations of rKLK10, followed by TNF α treatment (5 ng/mL for 4h). Treatment with rKLK10 significantly inhibited monocyte adhesion to ECs in a concentration-dependent manner (FIG. 2B). Furthermore, treatment with rKLK10 significantly
10 inhibited mRNA and protein expression of the pro-inflammatory adhesion molecules VCAM1 and ICAM1 (FIGS. 2C-2G), as well as the phosphorylation and nuclear localization of NF κ B in response to TNF α (FIG. 12). The anti-inflammatory effect of rKLK10 was lost if rKLK10 was heated, highlighting the importance of the enzymatic activity or native conformation of KLK10.

The effect of KLK10 on the endothelial inflammatory response was assessed as measured
15 by monocyte adhesion under flow-conditions *in vitro* and *in vivo*. rKLK10 treatment inhibited OS-induced monocyte adhesion in HAECs (FIG. 2H). In contrast, siRNA-mediated knockdown of KLK10 significantly increased monocyte adhesion under LS conditions (FIG. 2I; FIG. 11). The effect of rKLK10 on endothelial inflammation was tested in naturally flow-disturbed LC of the aortic arch in mice. Treatment with rKLK10 *in vivo* (intravenous injection every 2 days for 5 days
20 at 0.6mg/kg) dramatically reduced VCAM1 expression in the *d-flow* (LC) region in the aortic arch of these mice (FIGS. 2J-2K). A dose-dependent effect of rKLK10 on VCAM1 expression was observed (FIG. 14). FIG. 13A and FIG. 13B show the change in VCAM1/ICAM1 mRNA expression by KLK10 treatment on TNF α -induced inflammation by qPCR. These results demonstrate that either KLK10 overexpression using plasmids or rKLK10 treatment protects
25 against EC inflammation both *in vitro* and *in vivo* under TNF α or *d-flow* conditions, whereas KLK10 downregulation under *d-flow* conditions or KLK10 knockdown using siRNA induces inflammation.

It was tested whether rKLK10 treatment can similarly reduce the permeability barriers of ECs. Thrombin treatment, as a positive control, increased the permeability of HAECs as measured
30 by increased binding of fluorescently labeled (FITC)-Avidin to biotin-gelatin as reported previously (Dubrovskiy O, et al. *Lab. Invest.* 2013, 93:254-263). Overnight rKLK10 pre-treatment prevented the permeability increase induced by thrombin in HAECs (FIGS. 3A-3B). Similarly, rKLK10 reduced the permeability induced by OS (FIGS. 3C-3D), further demonstrating the protective role of KLK10 in endothelial inflammation and barrier function.

Example 4

Treatment with rKLK10 inhibits atherosclerosis in ApoE^{-/-} mice

In view of the anti-inflammatory and barrier protection effect of rKLK10 in ECs, it was tested whether atherosclerosis development could be prevented by treating mice with rKLK10. The PCL model of atherosclerosis was used to induce atherosclerosis rapidly in a flow-dependent manner in hyperlipidemic ApoE^{-/-} mice fed with a high-fat diet. Injection with rKLK10 (twice per week at 0.6 mg/kg for 3 weeks post-PCL surgery) significantly reduced atherosclerosis development and macrophage accumulation in the LCA (FIGS. 4A-4E). The rKLK10 treatment showed no effect on plasma levels of total, LDL, and HDL cholesterol and triglycerides (FIGS. 4F-4I). Thus, rKLK10 had an anti-atherogenic effect *in vivo*.

Example 5

Ultrasound-mediated overexpression of KLK10 inhibits atherosclerosis in ApoE^{-/-} mice

It was asserted whether KLK10 overexpression using a plasmid vector could also inhibit atherosclerosis *in vivo*. Either KLK10 plasmid (pCMV-Igκ-KLK10-T2A-Luc) or luciferase plasmid (pCMV-Luc) as a control was injected along with microbubbles into the hind-limbs of ApoE^{-/-} mice and mouse legs were sonoporated with ultrasound as previously described (Liu R, et al. *ACS Nano*. 2019, 13:5124-5132; Borden MA, et al. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control*. 2005, 52:1992-2002; Shapiro G, et al. *J Control Release*. 2016, 223:157-164). The plasmid injection and sonoporation process was repeated 10 days later to ensure sustained protein expression for the duration of the study. The mice were imaged three weeks later for bioluminescence and sacrificed for atherosclerosis studies.

Bioluminescence imaging showed that all mice expressed luciferase in the hind-limbs at the conclusion of the study, indicating successful overexpression of the plasmids (FIG. 5A). Atherosclerotic plaque formation in the LCA was significantly reduced in the KLK10 overexpressing mice compared to the luciferase control (FIG. 5B). Further assessment of the carotid artery sections by histochemical staining with hematoxylin and eosin (FIGS. 5D-5E) and Oil-red-O (FIG. 14) showed decreased plaque area and lipid accumulation, respectively, in the LCA of KLK10 overexpressing mice. Although circulating KLK10 levels in the mice could not be measured due to lack of suitable antibodies for ELISA, higher levels of KLK10 staining were observed at the endothelial layer in the LCA and RCA (FIGS. 5F-5G), as well as in lung tissue samples as shown by western blot (FIGS. 5H-5I). These results demonstrate that treatment with KLK10 by either rKLK10 or KLK10 expression vector can inhibit atherosclerosis development in

ApoE^{-/-} mice.

Example 6

KLK10 inhibits endothelial inflammation in a protease activated receptor-1/2 (PAR1/2)-dependent manner, but without directly cleaving the receptors

PARs (1, 2 and 4) have been shown to mediate the effects of some KLKs (e.g. KLK5, 6, and 14) (Oikonomopoulou K, et al. *J Biol Chem.* 2006, 281:32095-32112; Oikonomopoulou K, et al. *Biol Chem.* 2006, 387:817-824). Therefore, whether the anti-inflammatory effect of KLK10 could be mediated by PARs was assessed. Since the gene array and qPCR data showed that mouse artery ECs express PAR1 and PAR2, but not PAR3 and PAR4 (Nam D, et al. *Am. J. Physiol-Heart C.* 2009, 297:H1535-H1543; Ni C-W, et al. *Blood.* 2010, 116:e66-e73), whether PAR1 and PAR2 mediate the KLK10 effect in ECs under OS condition was assessed using two independent approaches: specific pharmacological inhibitors and siRNAs. HUVECs were first exposed to OS for 16 h to induce an endothelial inflammatory response, and treated with either the PAR1 inhibitor (SCH79797, 0.1μM) or PAR2 inhibitor (FSLRY-NH₂, 10μM). Both PAR inhibitors prevented the anti-inflammatory effect of KLK10 (FIG. 6A), suggesting the role of PAR1/2 in mediating the KLK10 effect. As a more specific approach, siRNAs were used for PAR1 or PAR2, which specifically knocked-down PAR1 and PAR2, respectively (FIG. 15). The siRNAs for PAR1 and PAR2 also prevented the anti-inflammatory effect of KLK10, as shown by the monocyte adhesion assay (FIG. 6B). When both PAR1 and PAR2 were knocked down together by the siRNAs, no additive effect was observed. These results suggest that both PAR1 and PAR2 are involved in mediating the anti-inflammatory effect of KLK10.

Since PARs are activated by site-specific cleavage of their extracellular N-terminal domain (Coughlin SR. *J Thromb Haemost.* 2005, 3:1800-1814; Coughlin SR. *Nature.* 2000, 407:258-264), it was assessed whether KLK10 can also cleave PAR1 and PAR2 to further define the mechanism of action. This study used two independent approaches. First, a secreted alkaline phosphatase (SEAP) reporter fused to the N-terminal domain of either PAR1 or PAR2 (PAR1-AP or PAR2-AP) was expressed in HAECs as reported previously (Rana S, et al. *J Cell Biochem.* 2012, 113:977-984; Mosnier LO, et al. *Blood.* 2012, 120:5237-5246; Bae JS, et al. *J. Thromb. Haemost.* 2008, 6:954-961). As expected, treatment with thrombin (PAR1 agonist) increased activity of SEAP from the PAR1-AP, indicating the cleavage of the PAR1 N-terminus in response to the canonical agonist (FIG. 6C). Similarly, trypsin (PAR2 agonist) also increased the SEAP activity in PAR2-AP expressing HAECs. Unexpectedly, however, treatment with rKLK10 did not induce the SEAP activity, indicating that it did not cleave the PAR1 or PAR2. To ensure that rKLK10 was still able

to inhibit endothelial inflammation, monocyte adhesion was concomitantly measured under the same conditions and the expected anti-inflammatory effect was observed (FIG. 15).

Next, it was tested whether KLK10 overexpression using the plasmid vector could cleave PAR1-AP or PAR2-AP in HAECs. Consistent with the rKLK10, KLK10 overexpression also failed to cleave either PAR1-AP or PAR2-AP, while thrombin and trypsin were still able to cleave the receptors (FIG. 6D). The same result was obtained using human embryonic kidney cells.

Next, as an alternative independent approach, a peptide cleavage assay was carried out using the synthetic N-terminal peptides corresponding to amino acid sequence 22-102 of PAR1 (PAR1²²⁻¹⁰²) and 26-75 of PAR2 (PAR2²⁶⁻⁷⁵), which contain canonical cleavage-activation sites for known proteinase agonists (Coughlin SR. *J Thromb Haemost.* 2005, 3:1800-1814; Coughlin SR. *Nature.* 2000, 407:258-264). Again, as expected, thrombin and trypsin efficiently cleaved the PAR1 and PAR2 peptides, respectively, as demonstrated by Coomassie staining of the Tricine-SDS PAGE gel (FIG. 6E). However, rKLK10 failed to cleave the peptides. These results demonstrated that neither rKLK10 nor KLK10 overexpressed by the expression vector can cleave and activate PAR1 or PAR2 receptors.

KLK10 had no significant cleavage activity against PAR-AP constructs or synthetic PAR1/2 N-terminal peptides. Thus, rKLK10 enzymatic activity was assessed. rKLK10 was incubated with an FP-biotin serine proteinase Activity-Based Probe and performed Streptavidin-HRP western blotting. rKLK10 was labelled with FP-biotin (FIGS. 6F-6E), indicating that KLK10 is indeed an active serine proteinase. Labeling by the activity-based probe was reduced by competition with FP-alkyne or when rKLK10 was heat-inactivated, suggesting that its 3D conformation is necessary for its enzymatic activity and the anti-inflammatory effect. Taken together, these results demonstrate that KLK10 inhibits endothelial inflammation in a PAR1/2-dependent manner, but without directly cleaving the receptors.

Example 7

KLK10 expression is decreased in human coronary arteries with atherosclerosis

It was assessed whether KLK10 expression is altered in human coronary artery tissue sections with varying degrees of atherosclerotic plaques. KLK10 immunostaining demonstrated KLK10 expression was predominantly present in non-diseased arteries, but was significantly reduced in arteries with plaques (FIGS. 7A-7B). No statistically significant correlation was observed between the disease progression and KLK10 levels due in part to a small number of samples studied (n=10).

The studies described herein show that *s-flow* promotes, while *d-flow* inhibits, expression

and secretion of KLK10 in ECs *in vitro* and *in vivo*. This work shows that KLK10 can inhibit endothelial inflammation and endothelial barrier dysfunction and reduce migration and tube formation, but neither inhibits nor reduces apoptosis or proliferation. Treatment of ECs *in vitro* with rKLK10 or a KLK10 expression vector inhibited endothelial inflammation induced by *d-flow* or TNF α . Moreover, treatment with rKLK10 or overexpression of KLK10 by ultrasound-mediated gene delivery inhibited endothelial inflammation and atherosclerosis development *in vivo*. These studies also revealed that the anti-inflammatory effect of KLK10 is mediated in part by both PAR1 and PAR2, but surprisingly without their direct cleavage and activation by KLK10 itself. rKLK10 and plasmid-driven KLK10 expression had protective effects on endothelial inflammation, barrier function, and atherosclerosis.

KLK10 was initially identified as a functional tumor suppressor since its expression inhibits oncogenicity of breast cancer cells and is downregulated in breast, prostate, testicular, and lung cancer (Goyal J, et al. *Cancer Res.* 1998, 58:4782-4786; Liu X-L, et al. *Cancer Res.* 1996, 56:3371-3379; Hu J, et al. *Sci. Rep.* 2015, 5:17426; Luo LY, et al. *Br J Cancer.* 2001, 85:220-224; Zhang Y, et al. *Cancer Sci.* 2010, 101:934-940). KLK10 is overexpressed in ovarian, pancreatic, and uterine cancer (Luo L-Y, et al. *Cancer Res.* 2003, 63:807-811; Luo L-Y, et al. *Tumor Biol.* 2006, 27:227-34; Yousef GM, et al. *Tumour Biol.* 2005, 26:227-235; Dorn J, et al. *Thromb. Haemost.* 2013, 110:408-422; Tailor PD, et al. *Oncotarget.* 2018, 9:17876-17888).

Studies provided herein using PAR inhibitors and siRNAs (FIGS. 6A-6B) showed that PAR1 and PAR2 receptors mediate the anti-inflammatory effects of KLK10 in ECs. Since both PAR1 and PAR2 can be activated by KLKs 5, 6, and 14 (Oikonomopoulou K, et al. *J. Biol. Chem.* 2006, 281:32095-32112; Oikonomopoulou K, et al. *J. Biol. Chem.* 2006, 387:817-824), it was surprising that KLK10 was not able to cleave and activate the PARs directly in a study using several independent approaches (FIG. 6). This was demonstrated by SEAP-PAR1/2 or N-Luciferase-PARs 1/2 cleavage assay in response to either rKLK10 or the KLK10 expression vector, as well as the cleavage assays using the PAR1/2 peptides representing the canonical cleavage sites. Nonetheless, the activity-based probe analysis showed that KLK10 was indeed catalytically active. KLK10 lost its enzymatic activity and the anti-inflammatory effect upon heat-denaturation, indicating that 3D conformation plays a role in enzymatic activity and biological activity.

KLK10's anti-inflammatory effect in ECs does not require that KLK10 directly cleave PAR1/2 receptors. The results provided herein demonstrated that KLK10 is a flow-sensitive factor (flow-kine) that is upregulated by *s-flow* conditions and downregulated by *d-flow* conditions in ECs, and that KLK10 mediates the anti-inflammatory effects of *s-flow* in a PAR1/2-dependent manner. Further, KLK10 itself is an anti-atherogenic therapeutic.

Example 8**His-tagged recombinant Human KLK10 Protein Production and Purification**

Wild-type (**SEQ ID NO: 3**) and mutant (**SEQ ID NO: 4**) His-tagged KLK10 proteins were produced and purified. The base sequence for recombinant human KLK10 was obtained from the National Center for Biotechnology Information (NCBI) database. Ser299Ala and Asp223Ala mutations were introduced to remove catalytic activity and substrate binding ability, respectively, on the basis that these residues are known catalytic triad and substrate binding sites (S1 pocket) (Debela M. et al. *Biol. Chem.* 2016, 397(12): 1251-1264). His-6 tags (VDHHHHHH (**SEQ ID NO: 27**)) were also introduced at C terminus of wild-type and mutant KLK10 for purification. Thus, these recombinant human KLK10 proteins contain the same pre-, pro-, and mature protein sequences as the native human KLK10 protein followed by an additional His-6 Tag. Table 4 provides structural, molecular weight, and amino acid modification information for each KLK10 protein produced.

Table 4		hKLK10 Wild Type	hKLK10 Mutant Type
Human KLK10		UniProt Accession No. O43240, GENBANK® Accession No. NP_001070968.1, both as available on September 8, 2020	
Serine Protease Domain	Catalytic triad	His86, Asp137, Ser229	His86, Asp137, Ser229Ala
	Substrate binding site	Asp223, Pro224	Asp223Ala, Pro224
Tag		C-terminal His-tag	
Theoretical Molecular Weight		27.7 kDa	27.7 kDa

DNA synthesis was performed according to the sequence information. Each DNA sequence was cloned into the MarEX vector, see U.S. Patent No. 8,772,021, to Kim et al., July 8, 2014, incorporated herein by reference. MarEX vectors for expressing His-tagged wild-type or mutant KLK10 were transfected separately to Chinese hamster ovary (CHO) cells, and stably expressing CHO cells were enriched using methotrexate (MTX) selection. Each His-tagged, recombinant human KLK10 protein was purified via affinity chromatography using HisPur Ni-NTA Resin (Thermo Scientific). Purified proteins were resolved using SDS-PAGE under reducing (R) or non-reducing (NR) conditions and stained with Coomassie blue (FIGS. 16-17). The purified proteins were observed at the predicted molecular weights and sequences were confirmed through peptide mapping using mass spectrometry.

SEQ ID NO: 25 (the following sequences are shown in **SEQ ID NO: 25**: Nhe I (GCTAGC)

- ACC - hKLK10 Wild Type - 6X His - TGA – Pme I (GTTTAAAC)) and **SEQ ID NO: 26** (the following sequences are shown in SEQ ID NO: 26: Nhe I (GCTAGC) - ACC - hKLK10 Mutant - 6X His - TGA – Pme I (GTTTAAAC)) provide nucleotide sequences including wild-type human KLK10 with a C-terminal His-tag and mutant (Ser299Ala and Asp223Ala) human KLK10 with a C-terminal His-tag, respectively. These sequences were cloned into the pcDNA 3.4 TOPO vector (ThermoFisher Scientific) for constitutive expression in mammalian cells. The pcDNA 3.4 TOPO vector is an exemplary expression vector that can be used to express KLK10 in mammalian cells, but other commercially available expression vectors can also be used. The pcDNA3.4 Topo vector was used to clone the KLK10 DNA initially, and then the KLK10 DNA was transferred to the MarFX vector system for upscaling protein production.

Example 9

Fusion Protein Production and Purification

Fc domain-KLK10 fusion proteins were produced and purified. As in Example 8, the base sequence for recombinant human KLK10 was obtained from the National Center for Biotechnology Information (NCBI) database. Table 5 provides structural, molecular weight, and amino acid modification information for each fusion protein.

Table 5	human KLK10 monomeric Fc	human KLK10 dimeric Fc
Structure	hKLK10 - Hinge - CH2 -CH3 (monomeric Fc)	hKLK10 - Hinge - CH2 -CH3 (dimeric Fc)
Human KLK10	NCBI Accession No. O43240, incorporated by reference as available on September 1, 2020	
IgG4 Hinge Region	C226S, C229S	S228P
IgG4 CH2 Region	F234A, L235A, M252Y, S254T, T256E	F234A, L235A, M252Y, S254T, T256E
IgG4 CH3 Region	L351F, T366R, P395K, F405R, Y407E	Wild Type
Theoretical Molecular Weight	52.4 kDa	104.8 kDa

For transient expression, plasmids comprising monomeric (**SEQ ID NO: 11**) or dimeric (**SEQ ID NO: 13**) Fc-KLK10 fusion sequences were transfected into CHO cells. Fusion proteins were harvested after 12 days and purified using protein A affinity chromatography. Purified proteins were resolved using SDS-PAGE under reducing (R) or non-reducing (NR) conditions and stained with Coomassie blue (FIG. 18). Under NR conditions, the purified monomeric and dimeric Fc-KLK10 fusion proteins were observed at the predicted molecular weights, and the sequences were confirmed through peptide mapping using mass spectrometry. In contrast, under R

conditions, the cleaved forms were observed for both the monomeric and dimeric Fc-KLK10 fusion proteins.

Regardless of the presence of different cleaved forms of KLK10, similar results were observed as compared to the His-tagged KLK10 protein (See Examples 1, 3, and 6) in an *in vitro* monocyte adhesion assay (FIG. 20). In this assay, HAEC cells (80,000 cells per well) were grown in 12-well dishes to 100% confluence. The cells were pretreated with 0.1-10 ng/ml of rKLK10 WT, rKLK10 mut, rKLK10-monomeric IgG4 Fc, rKLK10-dimeric IgG4 Fc, or a heat-inactivated version thereof, in medium containing 2% FBS, and 16 hours later were treated with 5 ng/ml TNF α for 5 hours. Then, cells were washed and BCECF-AM ((2',7' Bis (2 Carboxyethyl) 5 (and 6))-labeled monocytes were added to the cultures for 30 min at 37°C. After the incubation, unbound cells were washed off and bound cells were counted by fluorescent microscopy. A minimum of 4 fields were counted for each experiment. The monocyte adhesion assay results showed that fusion proteins including wild-type (SEQ ID NO: 1) or mutant KLK10 (SEQ ID NO: 2) both exhibit anti-atherosclerotic effects *in vitro*. Selected Fc-KLK10 fusion proteins will be tested in *in vivo* anti-atherogenic efficacy analyses.

In view of the many possible embodiments to which the principles of our invention may be applied, it should be recognized that illustrated embodiments are only examples of the invention and should not be considered a limitation on the scope of the invention. Rather, the scope of the invention is defined by the following claims. We therefore claim as our invention all that comes within the scope and spirit of these claims.

We claim:

1. A method of treating atherosclerosis in a subject, comprising administering to the subject a therapeutically effective amount of a Kallikrein Related Peptidase 10 (KLK10) protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, thereby treating the atherosclerosis in the subject.
2. A method of decreasing arterial endothelial inflammation in a subject, comprising selecting a subject with atherosclerosis, and administering to the subject a therapeutically effective amount of a KLK10 protein, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK protein or the fusion protein, thereby decreasing arterial endothelial inflammation in the subject.
3. A fusion protein comprising a KLK10 protein and an Fc domain.
4. The method of claim 1 or claim 2, or the fusion protein of claim 3, wherein the fusion protein further comprises a His-tag.
5. The method of claim 1 or claim 2, or the fusion protein of claim 3 or claim 4, wherein the KLK10 protein comprises an amino acid sequence at least 90% identical to amino acids 43-276 of SEQ ID NO: 1 or amino acids 43-276 of SEQ ID NO: 2.
6. The method of claim 1 or claim 2, or the fusion protein of claim 3 or claim 4, wherein the KLK10 protein comprises an amino acid sequence at least 95% identical to amino acids 43-276 of SEQ ID NO: 1 or amino acids 43-276 of SEQ ID NO: 2.
7. The method of claim 1 or claim 2, or the fusion protein of claim 3 or claim 4, wherein the KLK10 protein comprises amino acids 43-276 of SEQ ID NO: 1 or amino acids amino acids 43-276 of SEQ ID NO: 2.
8. The method of claim 1 or claim 2, or the fusion protein of claim 3 or claim 4, wherein the KLK10 protein comprises:
 - a) an amino acid sequence at least 90% identical to amino acids 34-276 of SEQ ID NO: 1 or amino acids 43-276 of SEQ ID NO: 2; or

b) an amino acid sequence at least 90% identical to SEQ ID NO: 1 or SEQ ID NO: 2.

9. The method of any one of claims 1-2 or 4-8, or the fusion protein of any one of claims 3-8, wherein the fusion protein comprises a Fc domain.

10. The method or fusion protein of claim 9, wherein the Fc domain is a monomeric Fc domain.

11. The method or fusion protein of claim 10, wherein the Fc domain comprises an amino acid substitution at one or more of residues 226, 229, 234, 235, 252, 254, 256, 351, 366, 395, 405, or 407, and wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

12. The method or fusion protein of claim 10, wherein the Fc domain comprises an amino acid substitution at at least one of:

one or more of residues 226 and 229 of the Fc hinge region;

one or more of residues 234, 235, 252, 254, and 256 of the Fc CH2 region; and

one or more of residues 351, 366, 395, 405, and 407 of the Fc CH3 region,

wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

13. The method or fusion protein of claim 10, wherein the Fc domain comprises one or more of amino acid substitutions C226S, C229S, F234A, L235A, M252Y, S254T, T256E, L351F, T366R, P395K, F405R, or Y407E, wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

14. The method or fusion protein of claim 10, wherein the Fc domain comprises at least one of:

one or more of amino acid substitutions C226S and C229S of the Fc hinge region;

one or more of amino acid substitutions F234A, L235A, M252Y, S254T, and T256E of the Fc CH2 region; and

one or more of amino acid substitutions L351F, T366R, P395K, F405R, and Y407E of the Fc CH3 region,

wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

15. The method or fusion protein of claim 10, wherein the Fc domain comprises an amino

acid sequence at least 95% identical to SEQ ID NO: 7 or SEQ ID NO: 8.

16. The method or fusion protein of claim 10, wherein the Fc domain comprises the amino acid sequence of SEQ ID NO: 7 or SEQ ID NO: 8.

17. The method or fusion protein of claim 9, wherein the Fc domain comprises a dimerization domain.

18. The method or fusion protein of claim 17, wherein the Fc domain comprises an amino acid substitution at one or more of residues 228, 234, 235, 252, 254, and 256, and wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

19. The method or fusion protein of claim 17, wherein the Fc domain comprises an amino acid substitution at least one of:

residue 228 of the Fc hinge region; and

one or more of residues 234, 235, 252, 254, and 256 of the Fc CH2 region,

wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

20. The method or fusion protein of claim 17, wherein the Fc domain comprises one or more of amino acid substitutions S228P, F234A, L235A, M252Y, S254T, and T256E of the Fc region, and wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

21. The method or fusion protein of claim 17, wherein the Fc domain comprises at least one of the following substitutions:

S228P of the Fc hinge region; and

one or more of F234A, L235A, M252Y, S254T, and T256E of the Fc CH2 region,

wherein the numbering of the amino acids in the Fc domain is IMGT numbering.

22. The method or fusion protein of claim 17, wherein the Fc domain comprises an amino acid sequence at least 95% identical to SEQ ID NO: 9 or SEQ ID NO: 10.

23. The method or fusion protein of claim 17, wherein the Fc domain comprises the amino acid sequence of SEQ ID NO: 9 or SEQ ID NO: 10.

24. The method or fusion protein of claim 17, wherein the fusion protein comprises an amino acid sequence at least 95% identical to any one of SEQ ID NOs: 11-14.

25. The method of any one of claims 1, 2, or 4-24, comprising administering the nucleic acid molecule encoding the KLK protein or the fusion protein to the subject.

26. The method of claim 25, comprising administering to the subject a therapeutically effective amount of a plasmid or a viral vector comprising the nucleic acid molecule encoding the KLK10 protein or the fusion protein.

27. The method of claim 26, wherein the viral vector is an adeno-associated virus (AAV) vector.

28. The method of claim 27, wherein the AAV vector is an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAV10, AAV11, or AAV12 vector, or a hybrid of two or more AAV serotypes.

29. The method of any one of claims 1, 2 or 4-28, wherein the subject has atherosclerotic heart disease.

30. The method of any one of claims 1, 2, or 4-29, further comprising administering to the subject a therapeutically effective amount of an antiplatelet agent, an anticoagulation agent, a lipid regulating agent, a blood pressure regulating agent, an antioxidant, or a combination thereof.

31. The method of claim 30, wherein the lipid regulating agent is a statin, niacin, PCSK9-targeting drug, bile acid binding resin, or an HDL-cholesterol targeting drug.

32. The method of claim 30, wherein the antioxidant is N-acetylcysteine, vitamin C, beta carotene, or vitamin E.

33. The method of any one of claims 1, 2, or 4-32, wherein the method inhibits monocyte adhesion to blood vessels, inhibits inflammation in blood vessels, and/or protects the endothelial permeability barrier in blood vessels in the subject.

34. The method of any one of claims 1, 2, or 4-33, comprising administering the KLK10 protein, the fusion protein, or the nucleic acid molecule, locally to a vessel of the subject.

35. The method of claim 34, comprising administering the KLK10 protein, the fusion protein, or the nucleic acid molecule, in a stent.

36. The method of any one of claims 1, 2, or 4-35, wherein the subject has a stroke, peripheral artery disease or myocardial infarction.

37. A composition comprising a KLK10 protein comprising an amino acid sequence at least 90% identical to amino acids 43-276 of SEQ ID NO: 1 or amino acids 43-276 of SEQ ID NO: 2, a fusion protein comprising the KLK10 protein, or a nucleic acid molecule encoding the KLK10 protein or the fusion protein, for use in the method of any one of claims 1, 2, or 4-36.

38. A nucleic acid molecule encoding the fusion protein of any one of claims 3-24.

39. The nucleic acid molecule of claim 38, wherein the KLK10 protein is encoded by a nucleotide sequence at least 95% identical to SEQ ID NO: 6.

40. An expression vector comprising the nucleic acid molecule of claim 38 or 39 operably linked to a heterologous promoter.

41. The expression vector of claim 40, wherein the expression vector is a viral vector.

42. The expression vector of claim 41, wherein the viral vector is an adeno-associated virus (AAV) vector.

43. The expression vector of claim 42, wherein the AAV vector is an AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV9, AAV10, AAV11, AAV12 vector, or a hybrid of two or more AAV serotypes.

44. An isolated host cell transfected with the expression vector of any one of claims 40-43.

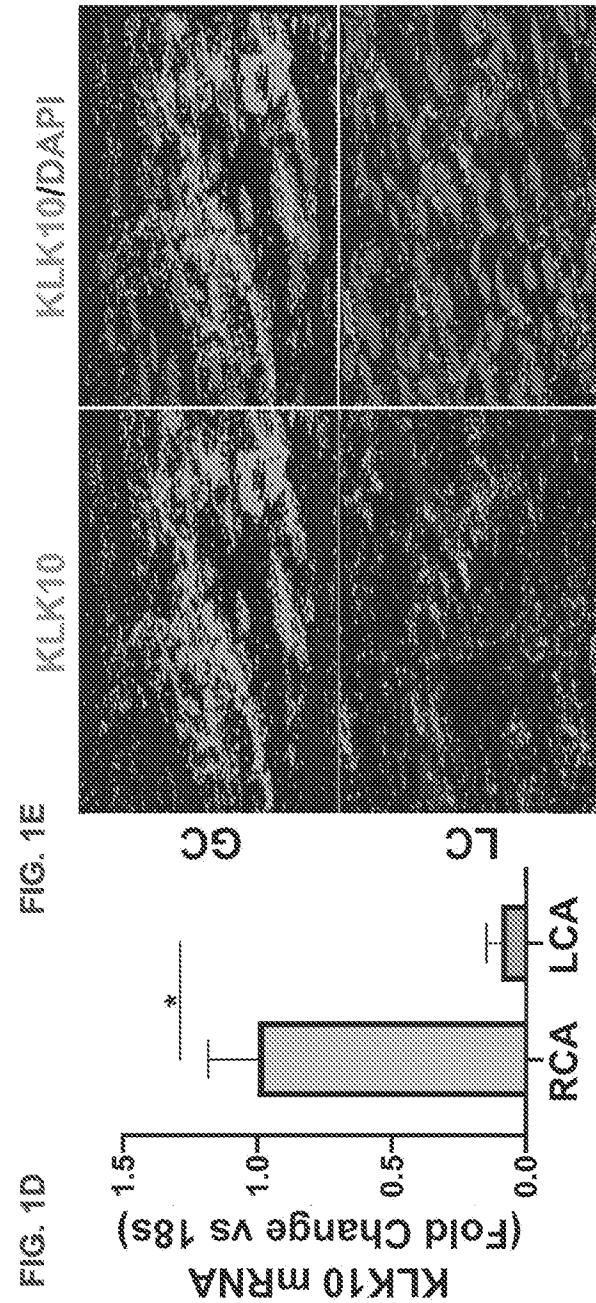
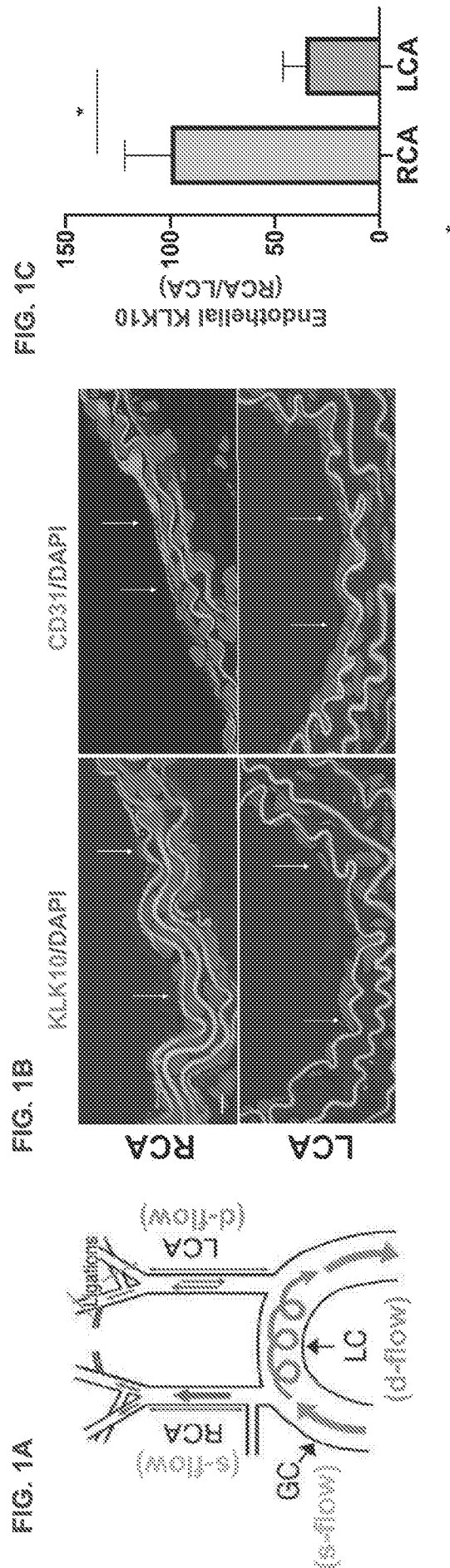


FIG. 1F

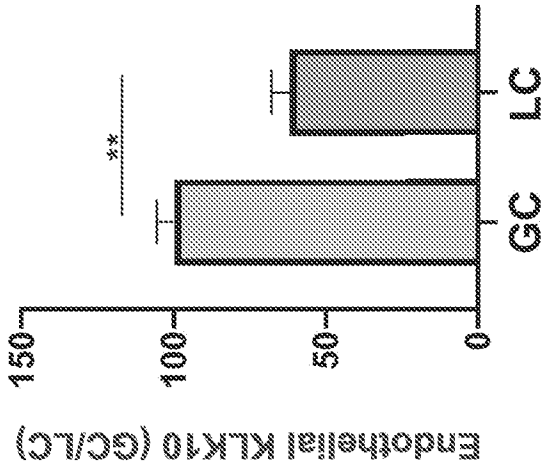


FIG. 1G

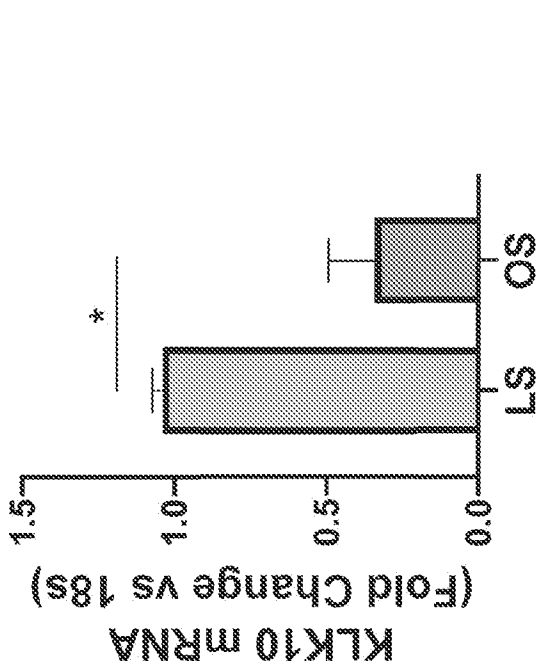


FIG. 1H

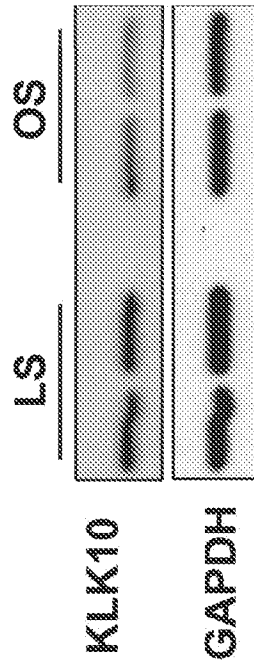


FIG. 1I

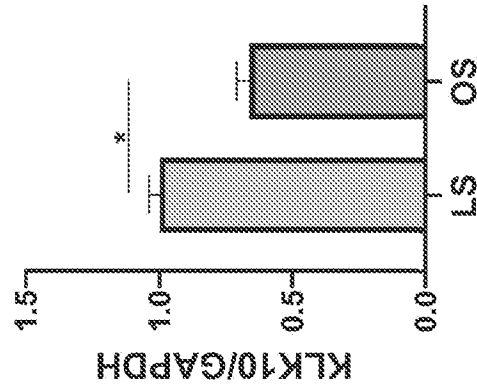


FIG. 1J

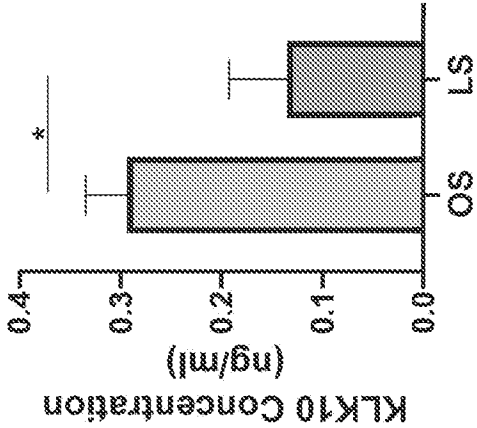


FIG. 2A

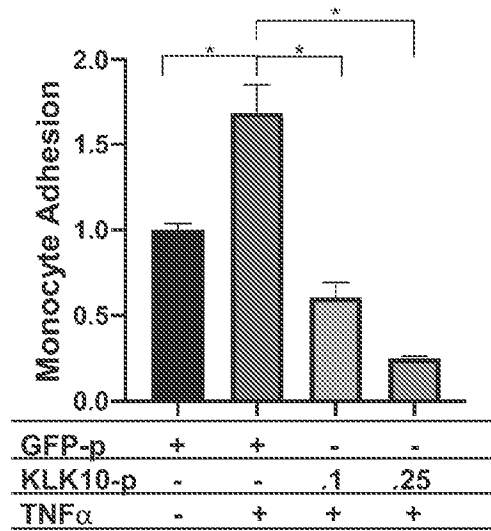


FIG. 2B

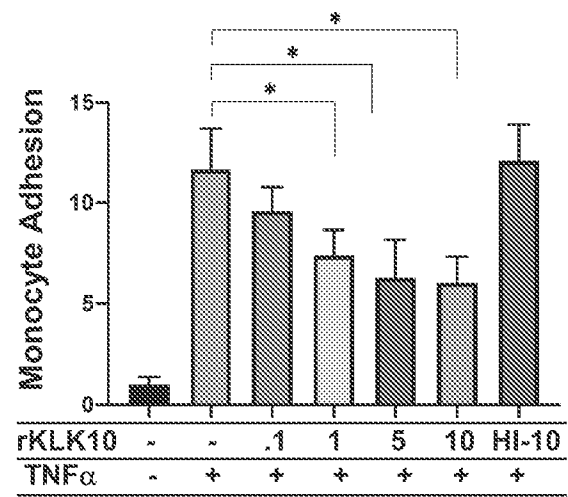


FIG. 2C

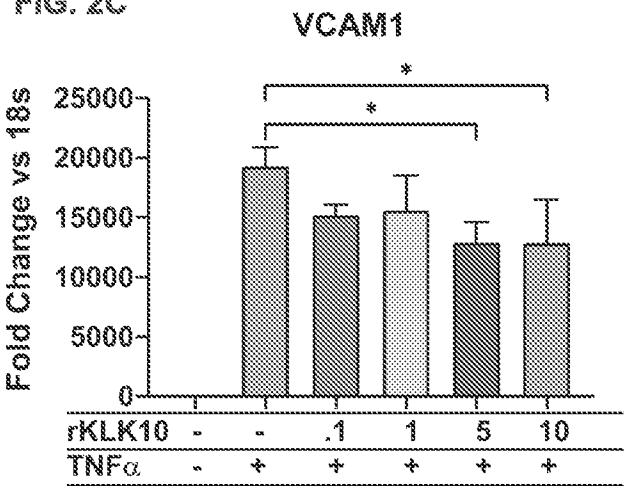


FIG. 2D

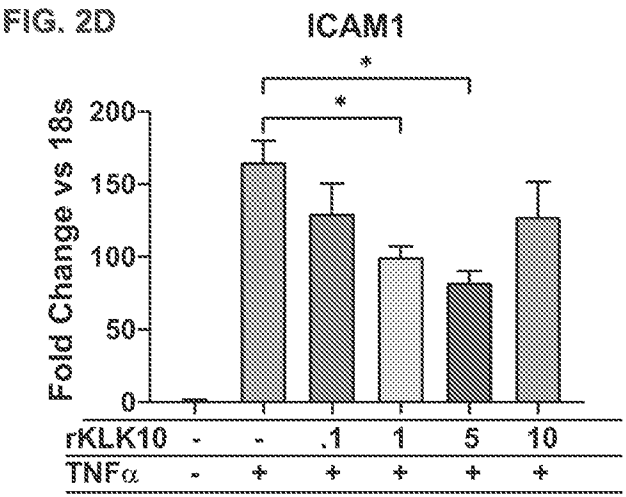


FIG. 2E

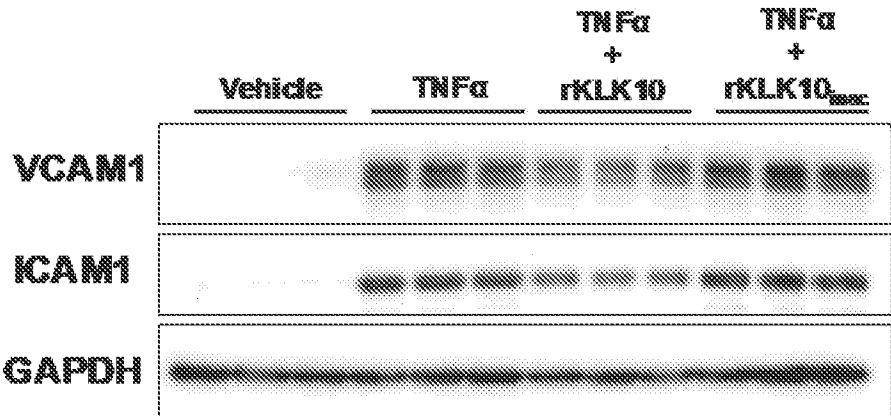


FIG. 2F

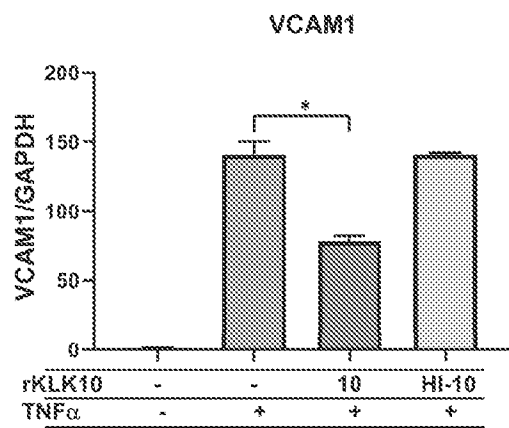


FIG. 2G

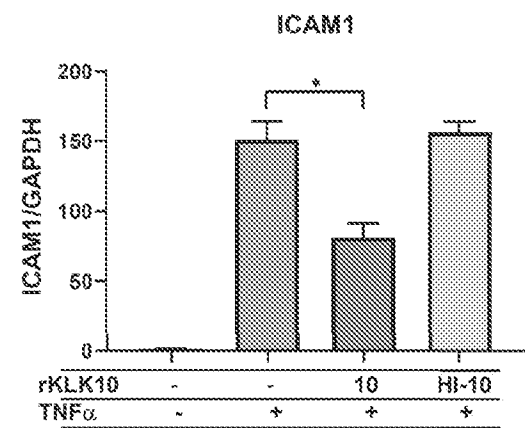


FIG. 2H

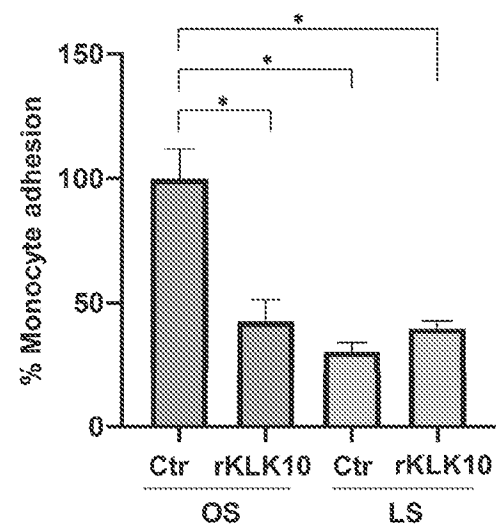


FIG. 2I

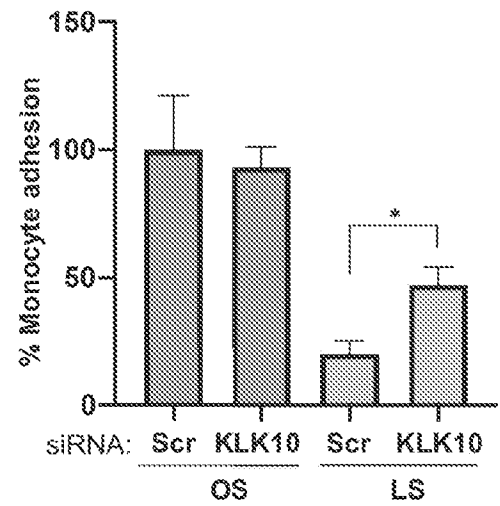


FIG. 2J

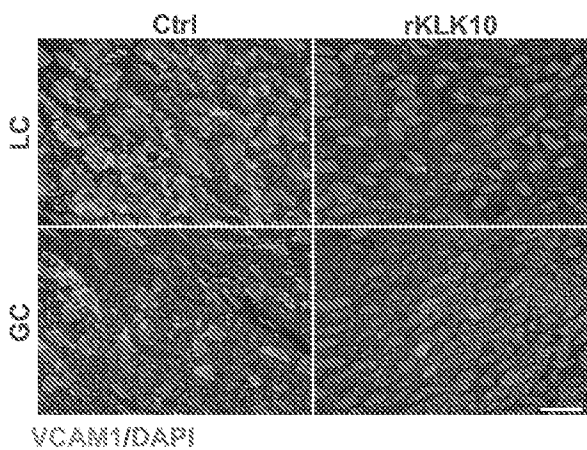


FIG. 2K

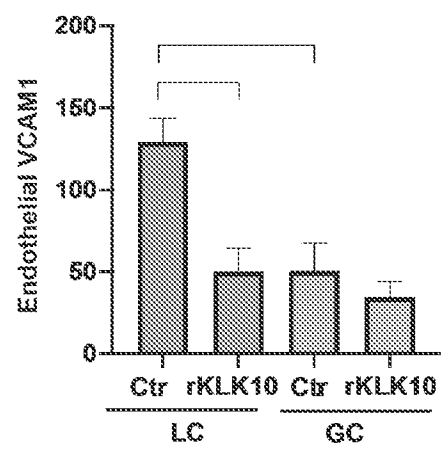


FIG. 3A

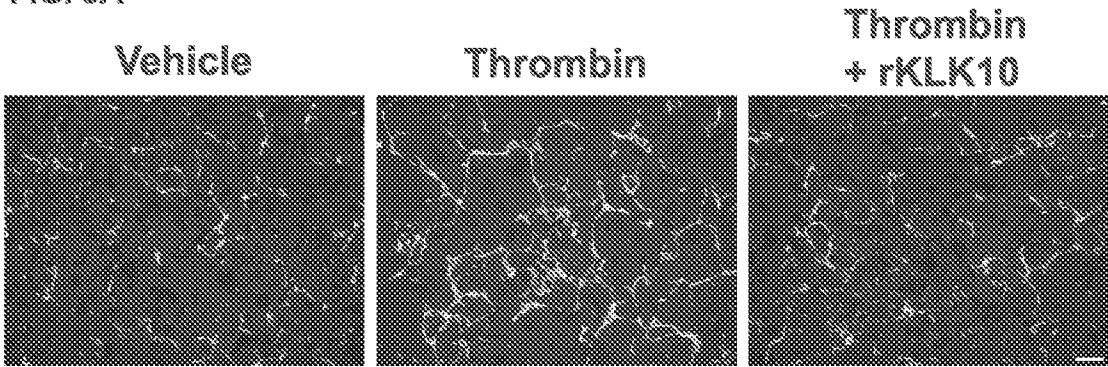


FIG. 3B

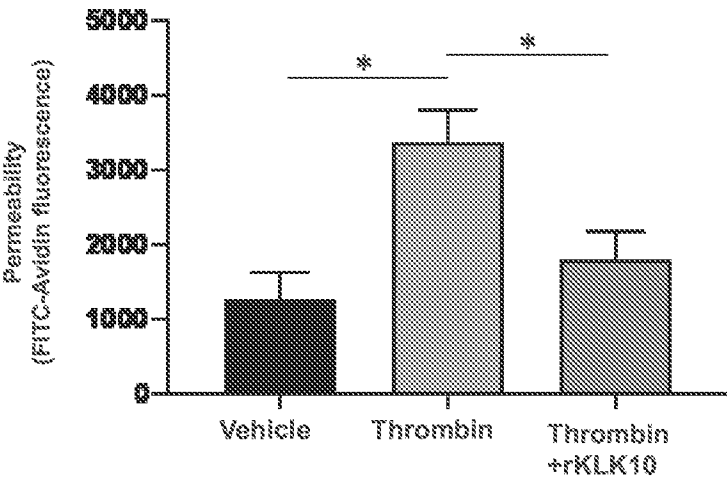


FIG. 3D

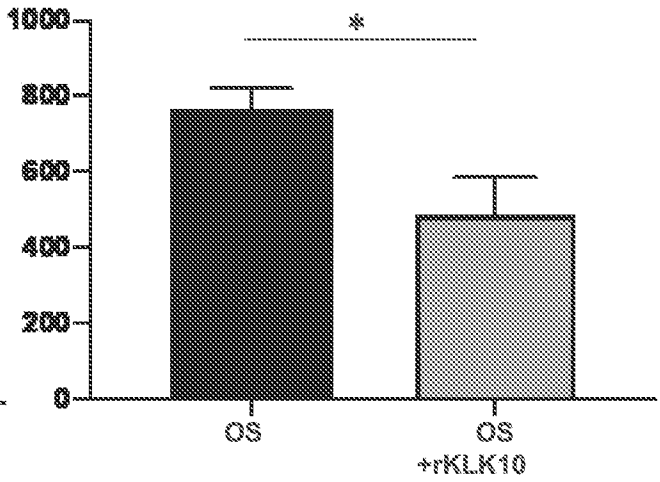


FIG. 3C

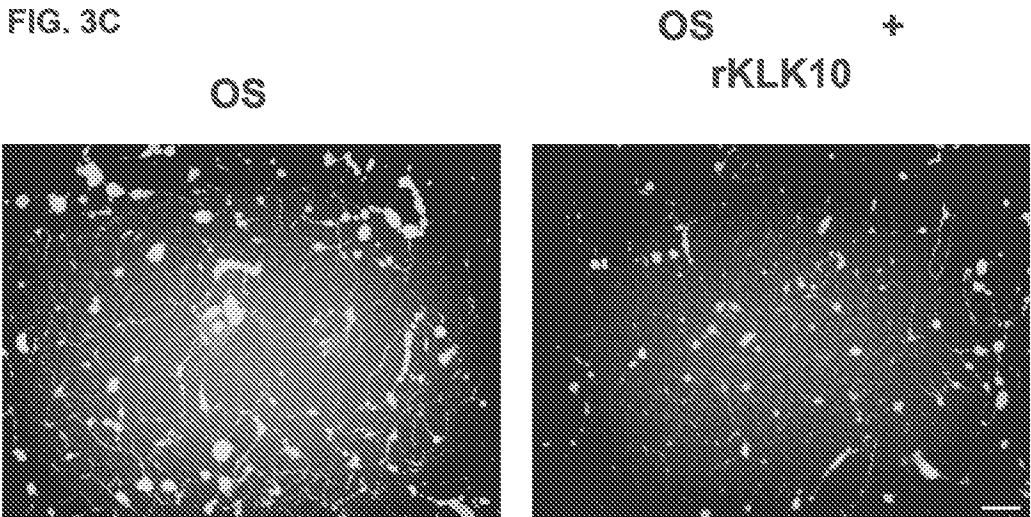


FIG. 4A



FIG. 4B

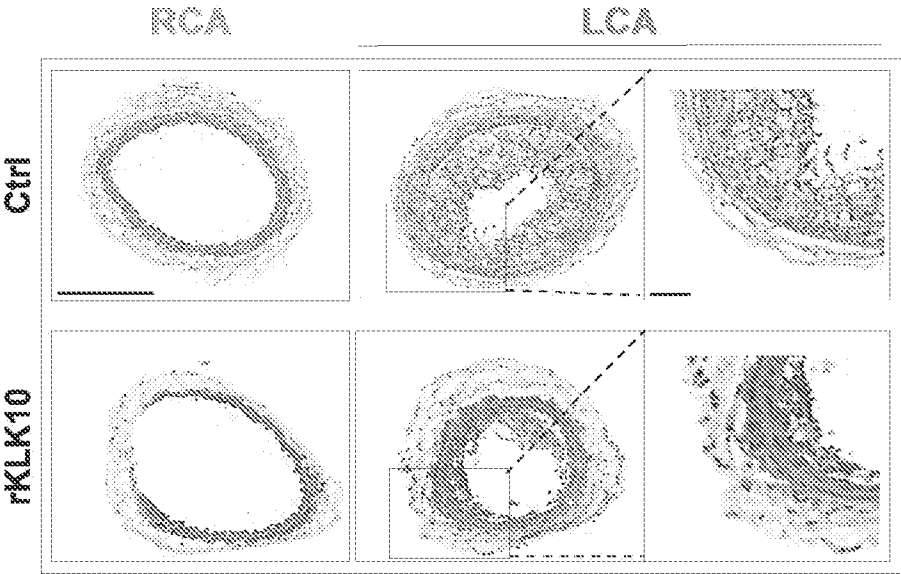


FIG. 4C

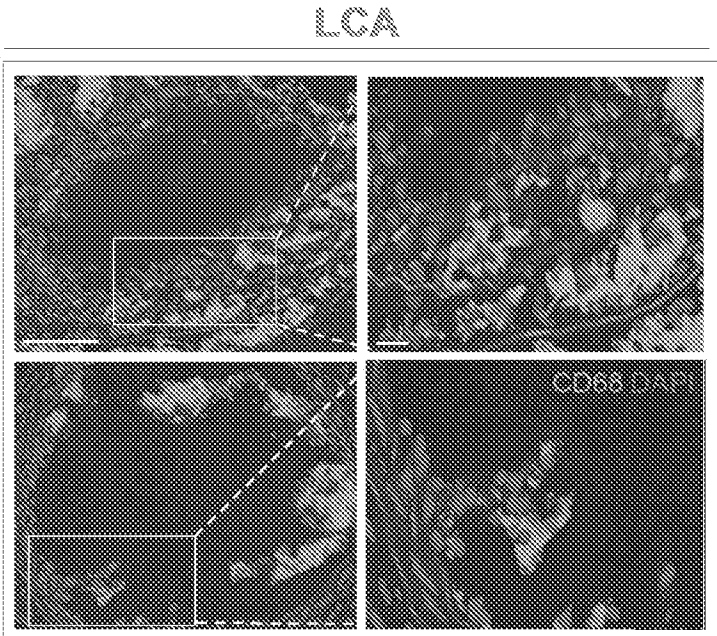


FIG. 4D

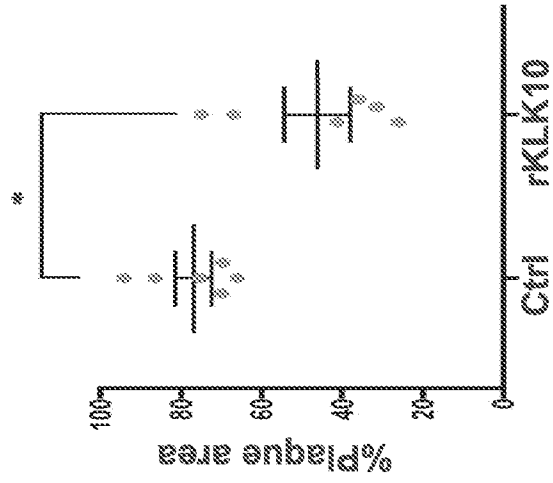


FIG. 4E

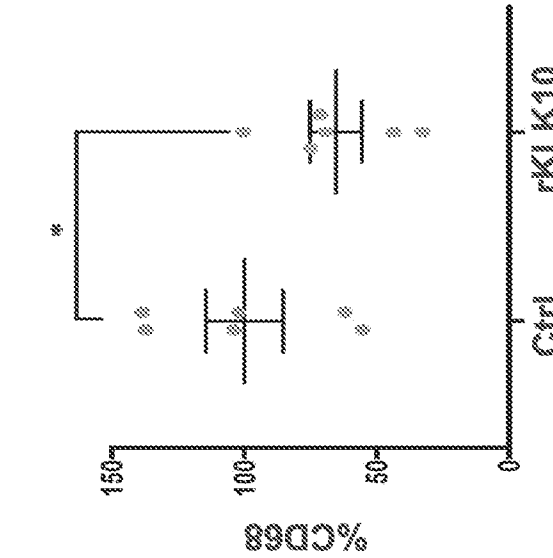


FIG. 4F

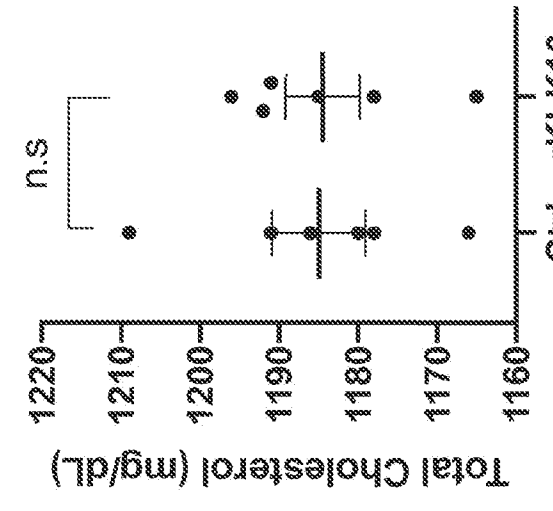


FIG. 4G

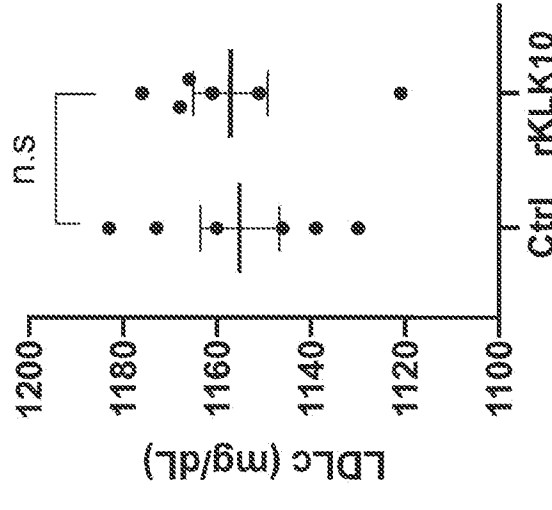


FIG. 4H

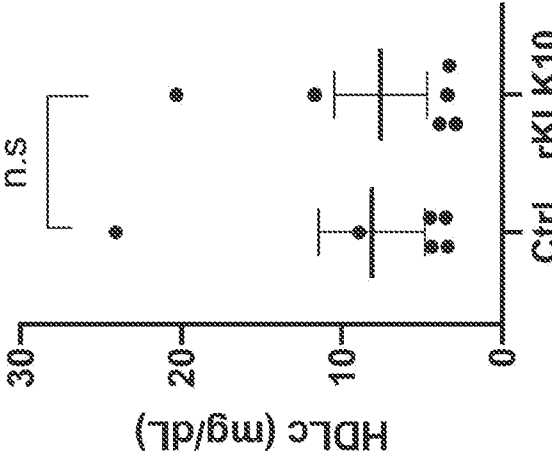
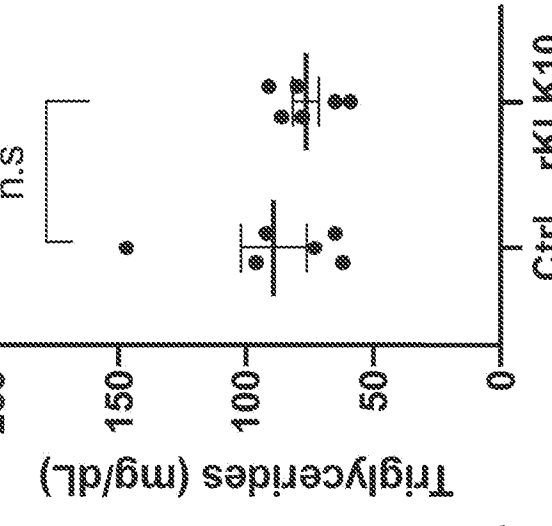
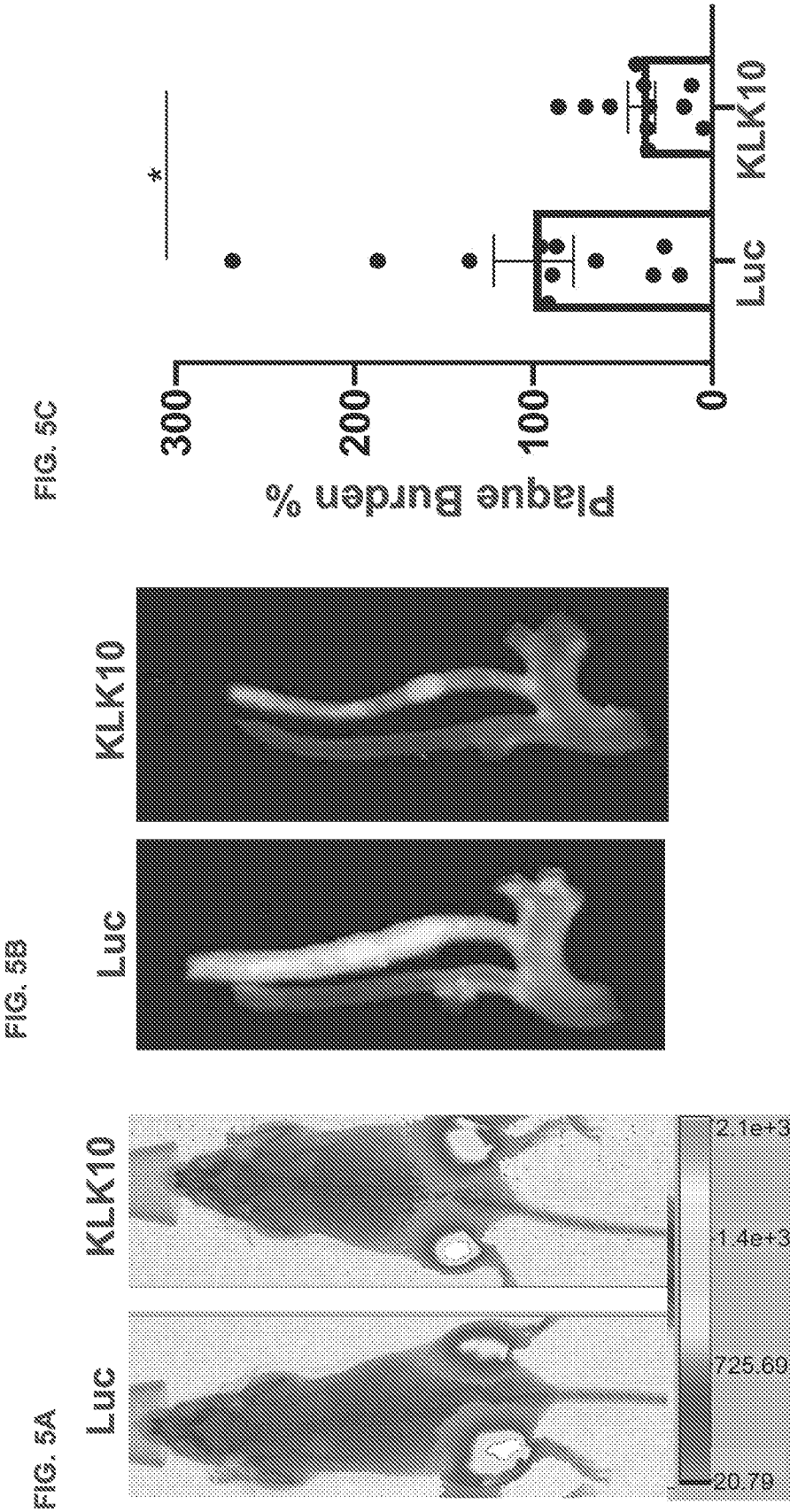


FIG. 4I





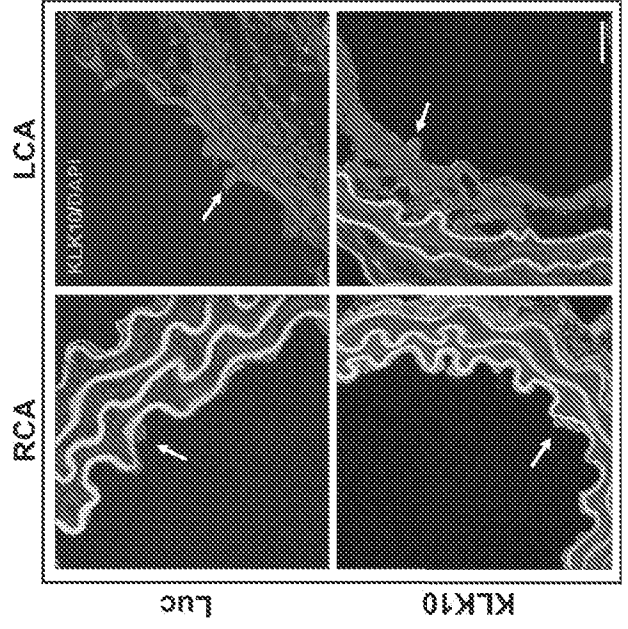
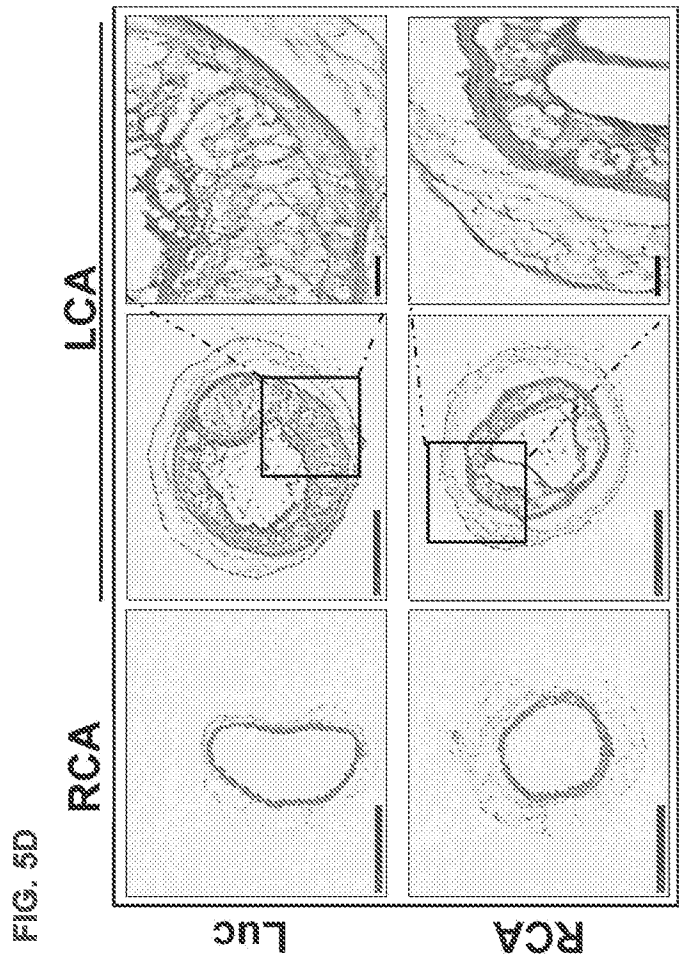
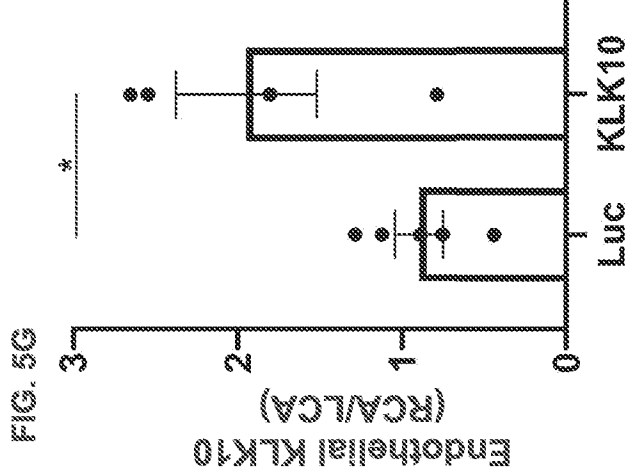
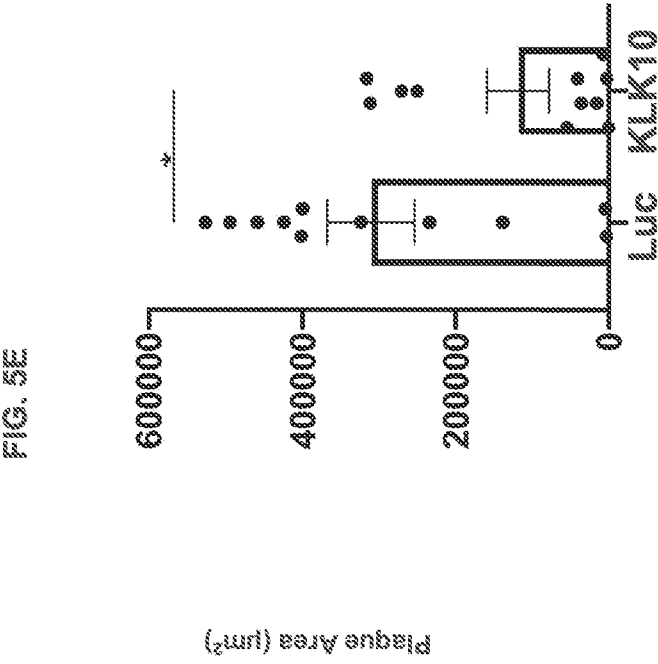


FIG. 5H

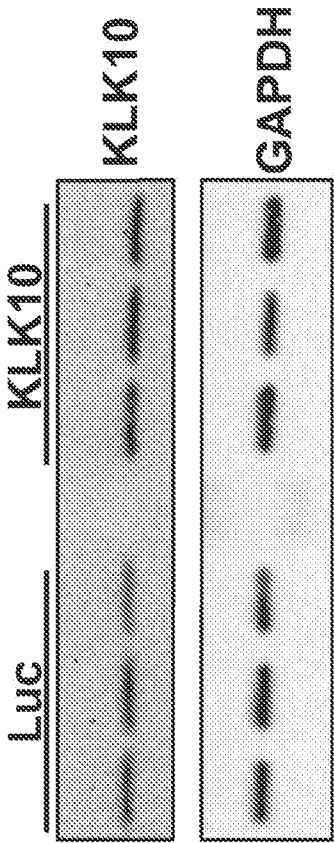


FIG. 5I

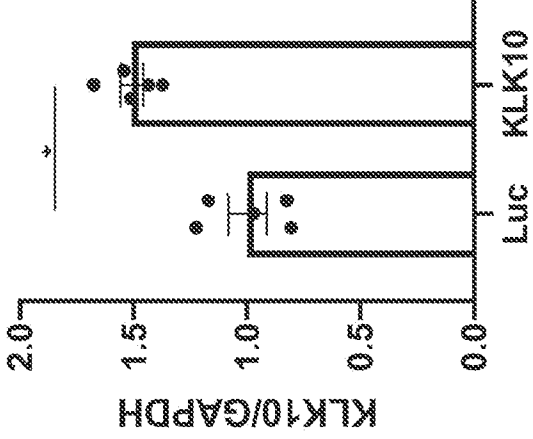


FIG. 5J

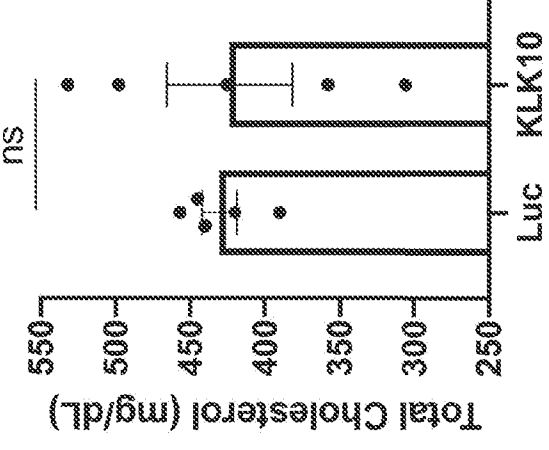


FIG. 5K

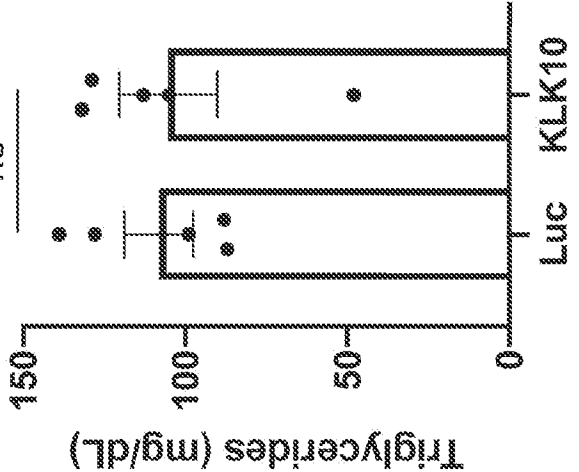


FIG. 5L

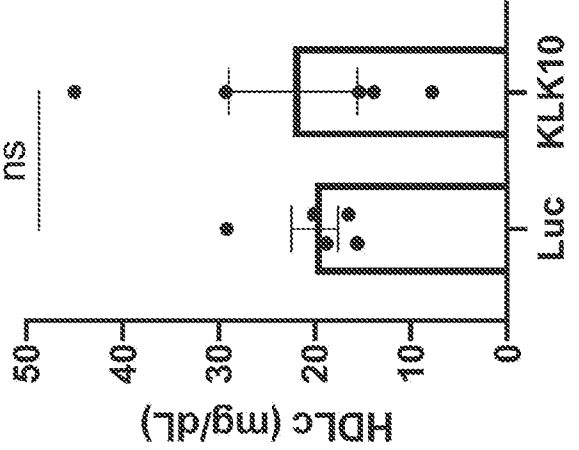


FIG. 5M

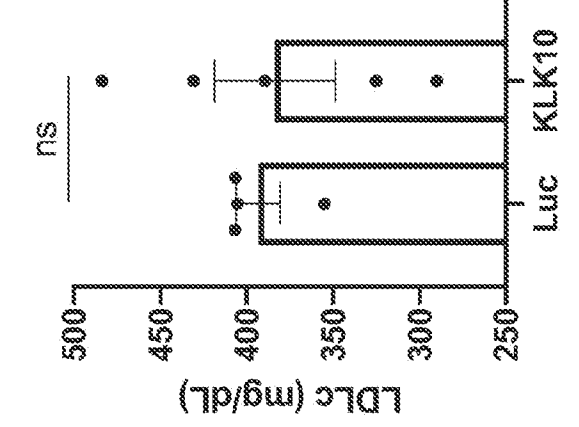


FIG. 5N

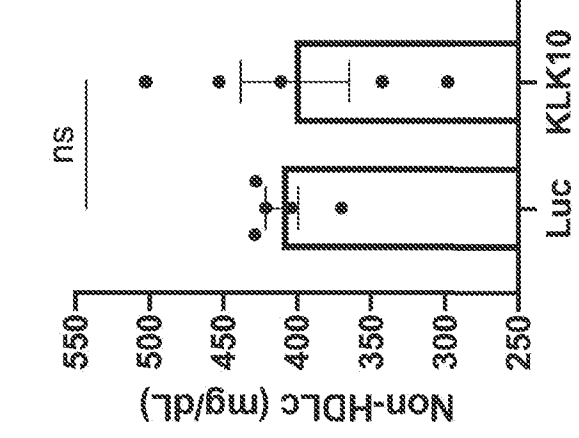


FIG. 6A

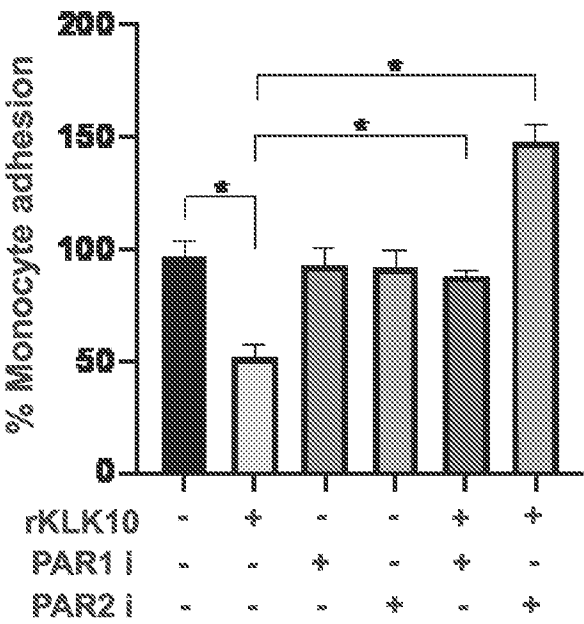


FIG. 6B

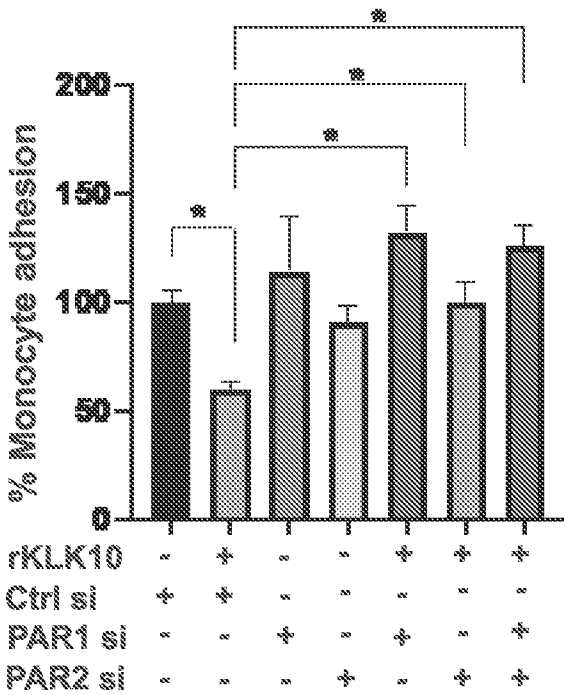


FIG. 6C

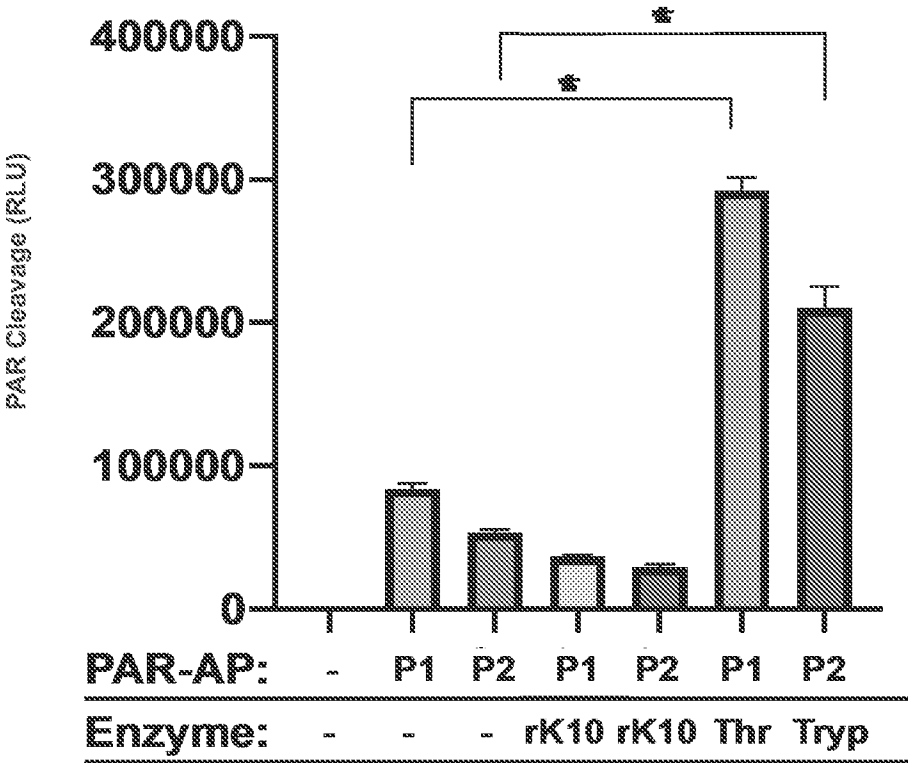


FIG. 6D

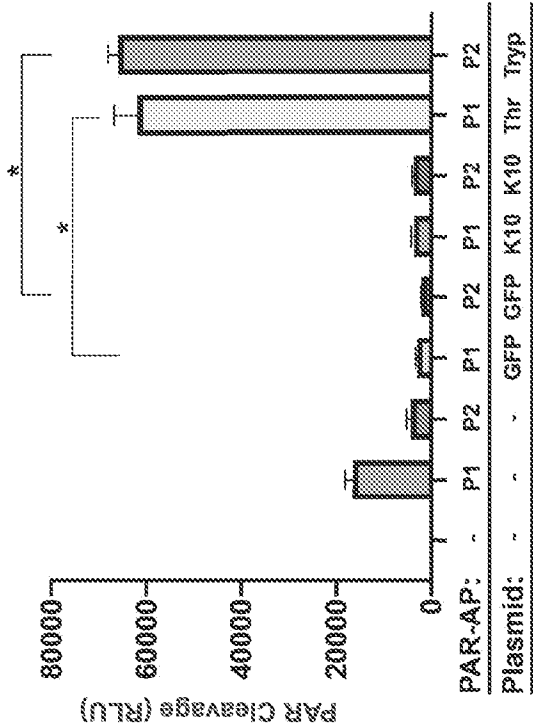


FIG. 6E

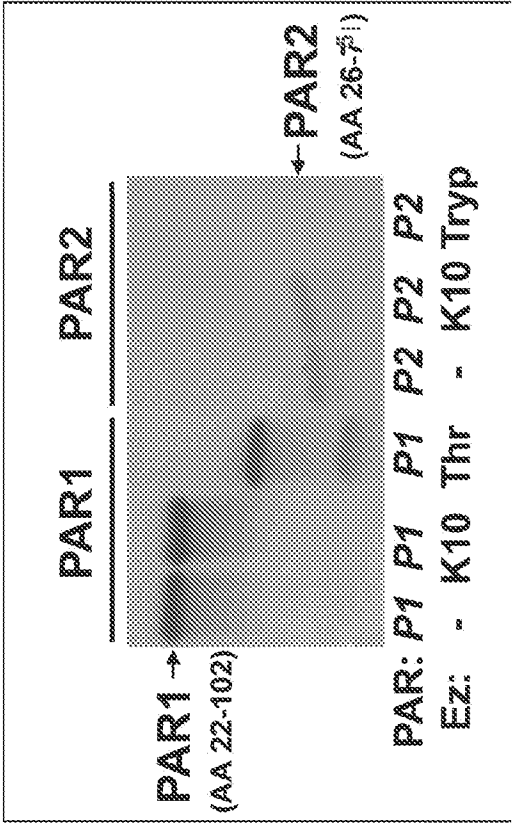


FIG. 6F

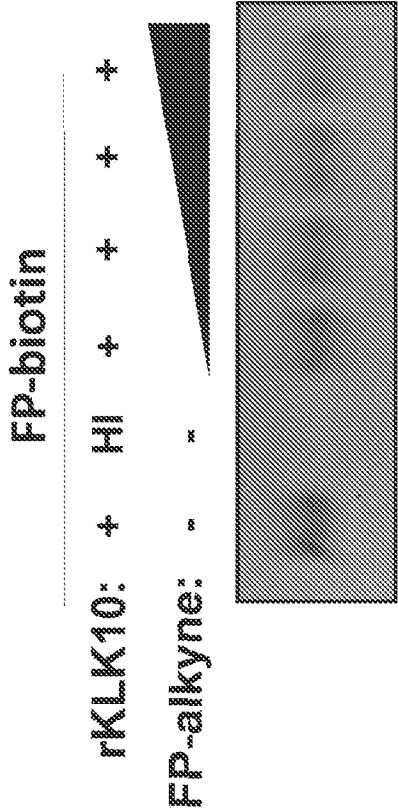


FIG. 6G

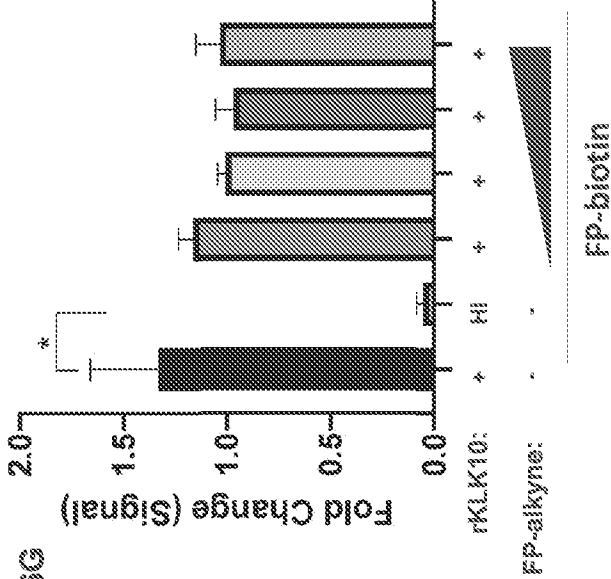


FIG. 7A

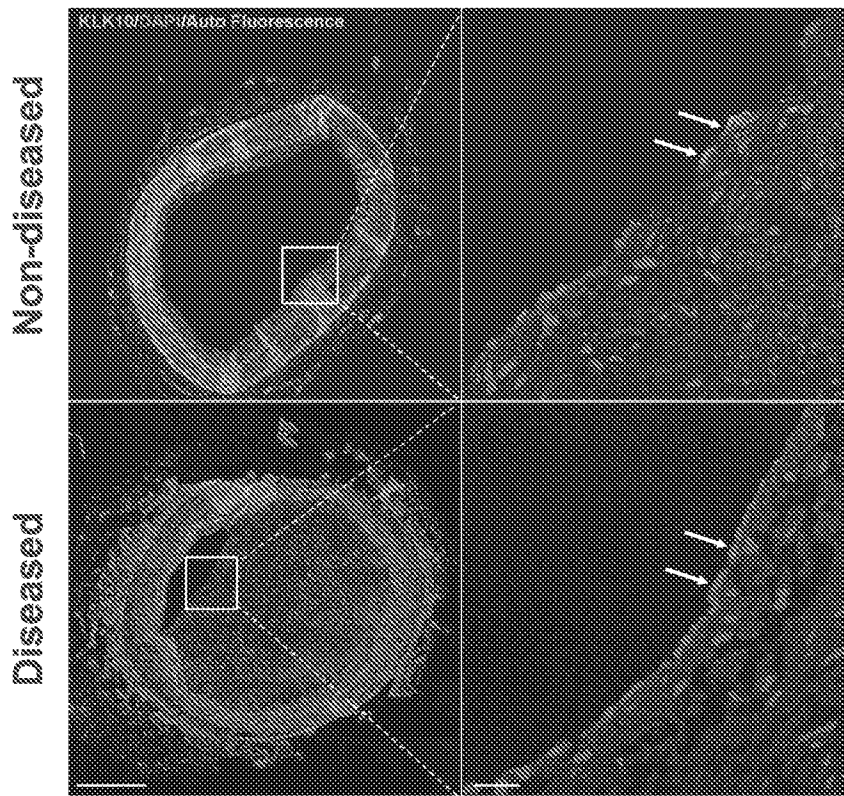


FIG. 7B

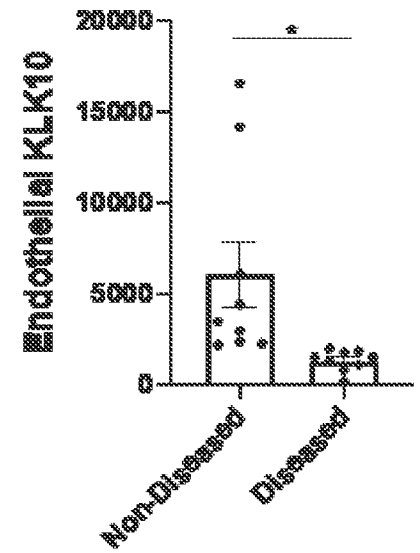


FIG. 8

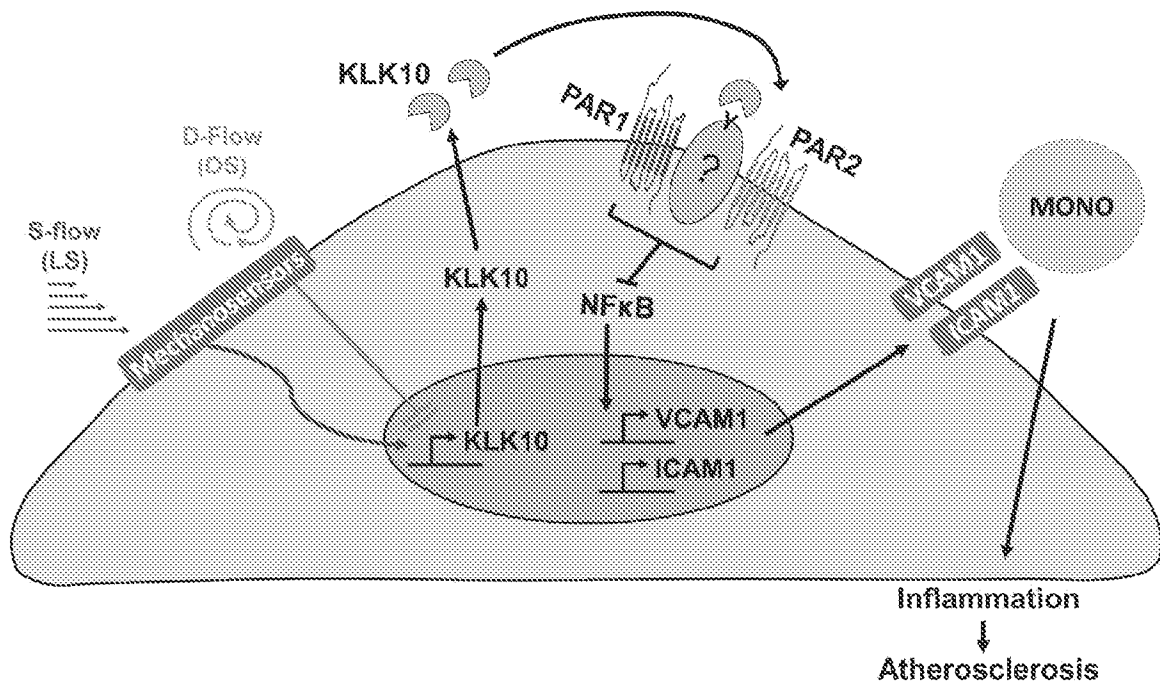


FIG. 9A

Migration

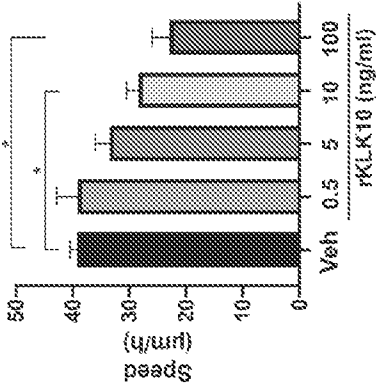


FIG. 9B

Apoptosis

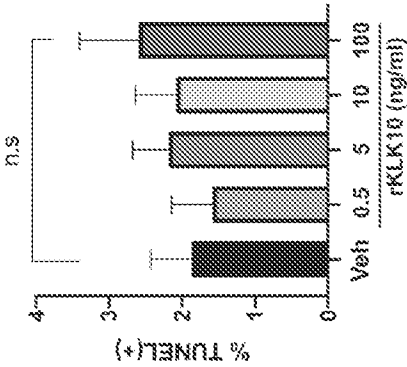


FIG. 9C

Proliferation

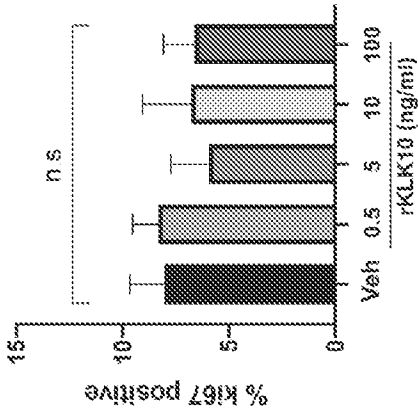


FIG. 9D

Tube Formation

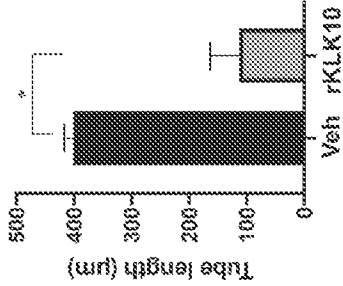


FIG. 10A

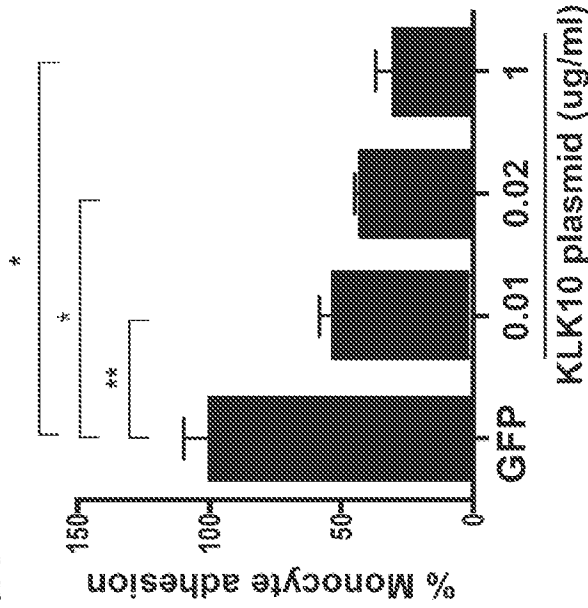


FIG. 10B

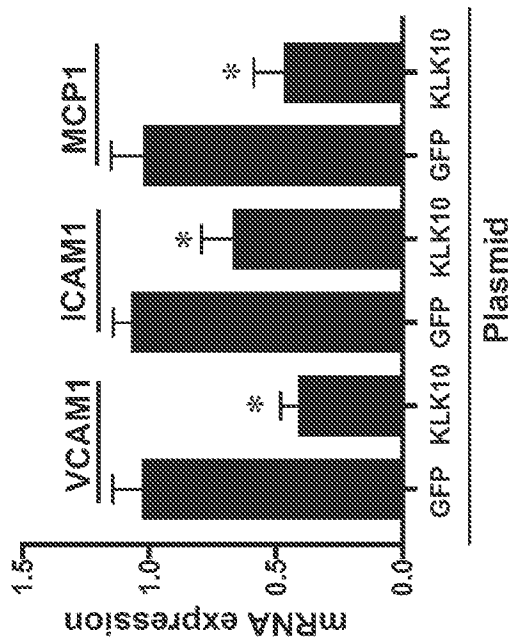


FIG. 10C

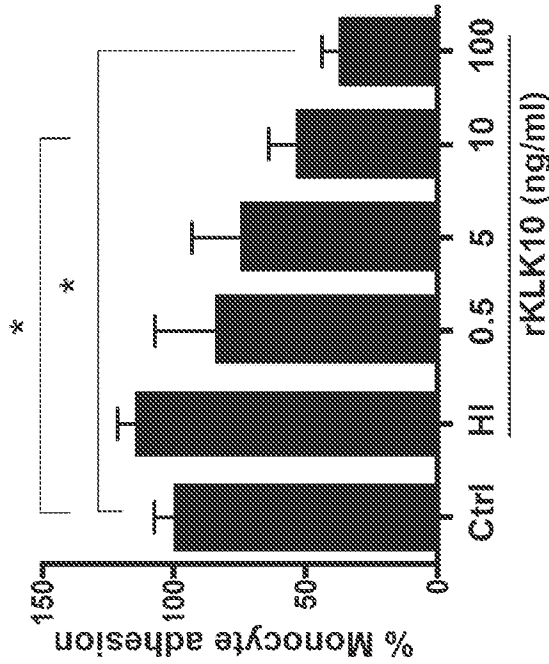


FIG. 10D

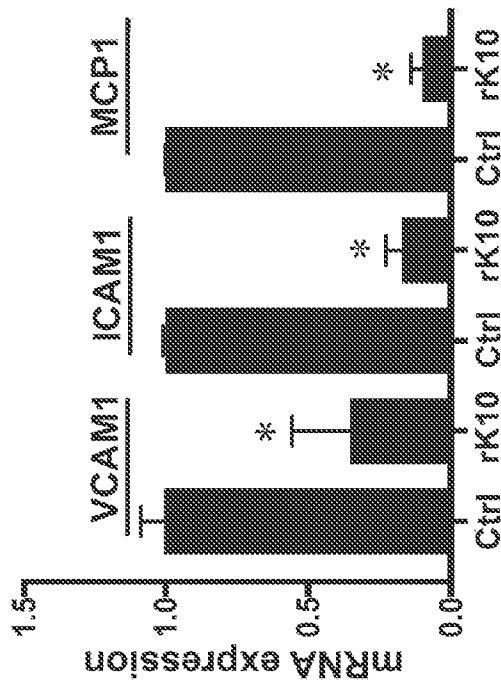


FIG. 11A

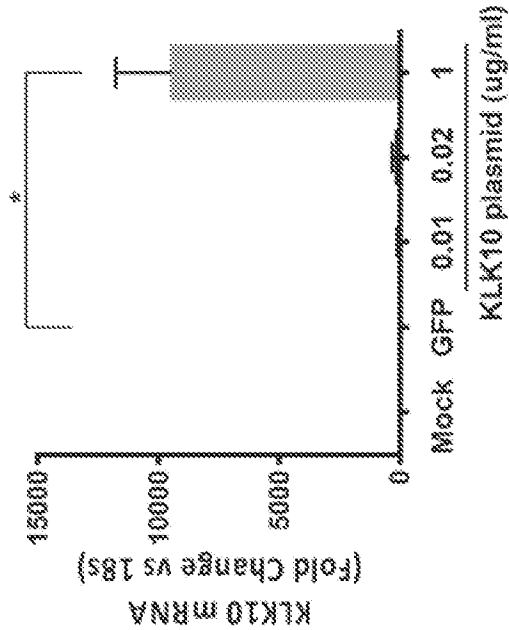


FIG. 11B

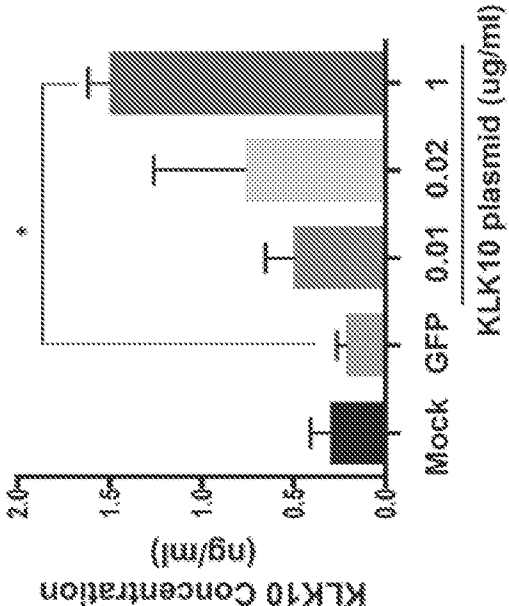


FIG. 11C

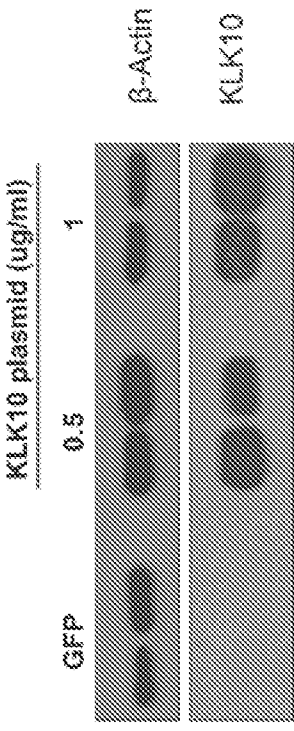


FIG. 11D

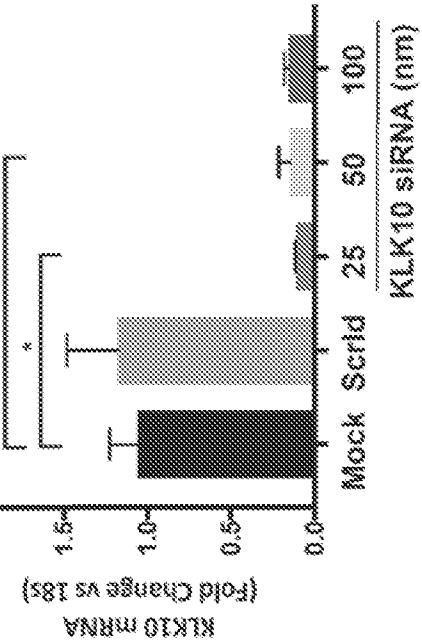


FIG. 12A

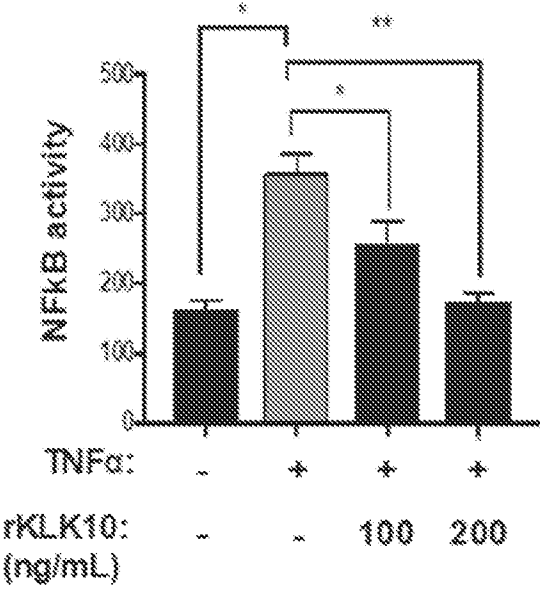


FIG. 12B

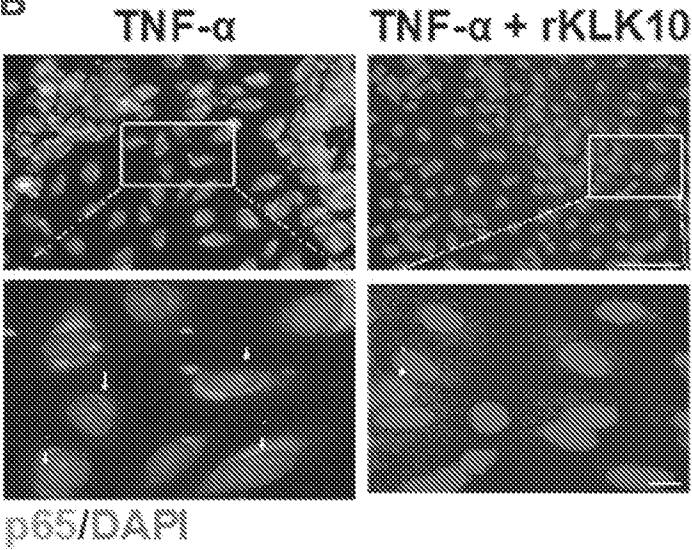


FIG. 12C

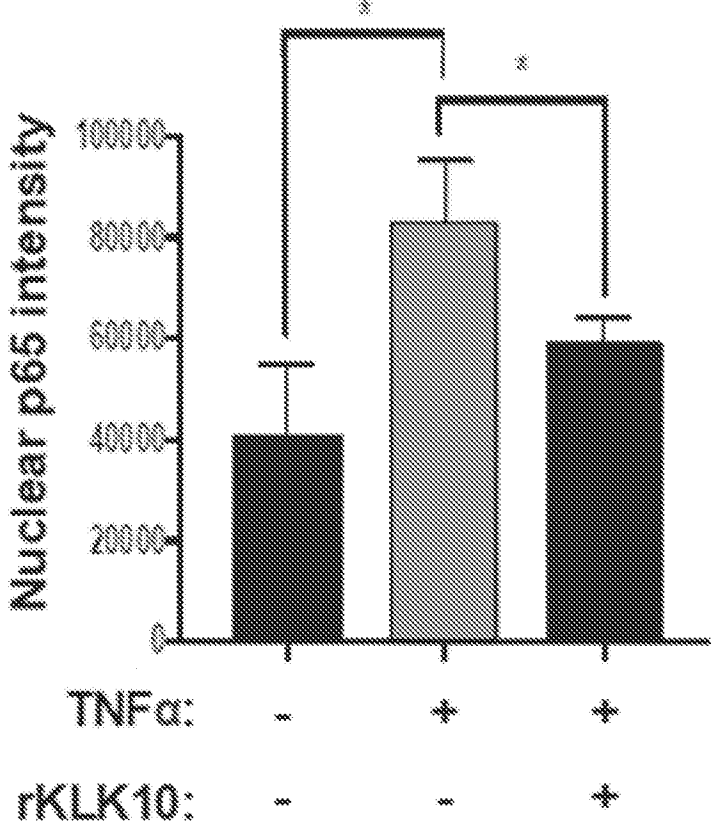


FIG. 12D

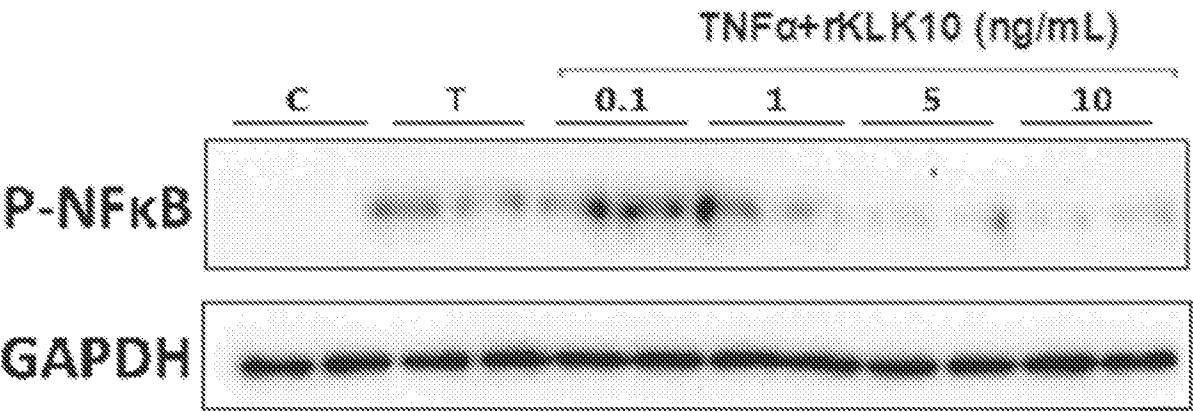


FIG. 12E

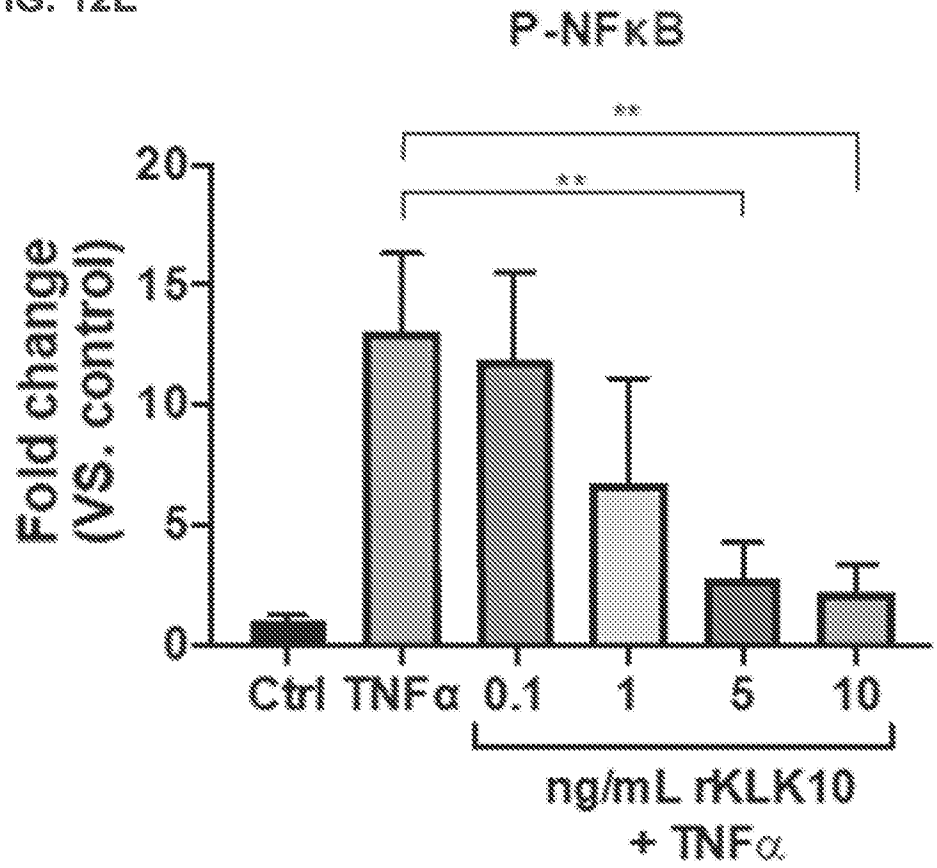


FIG. 13A

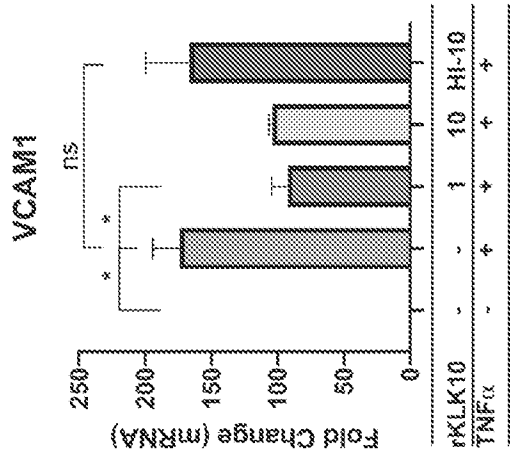


FIG. 13B

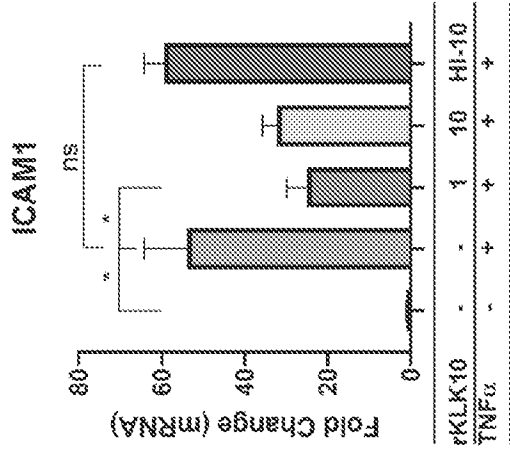


FIG. 14A

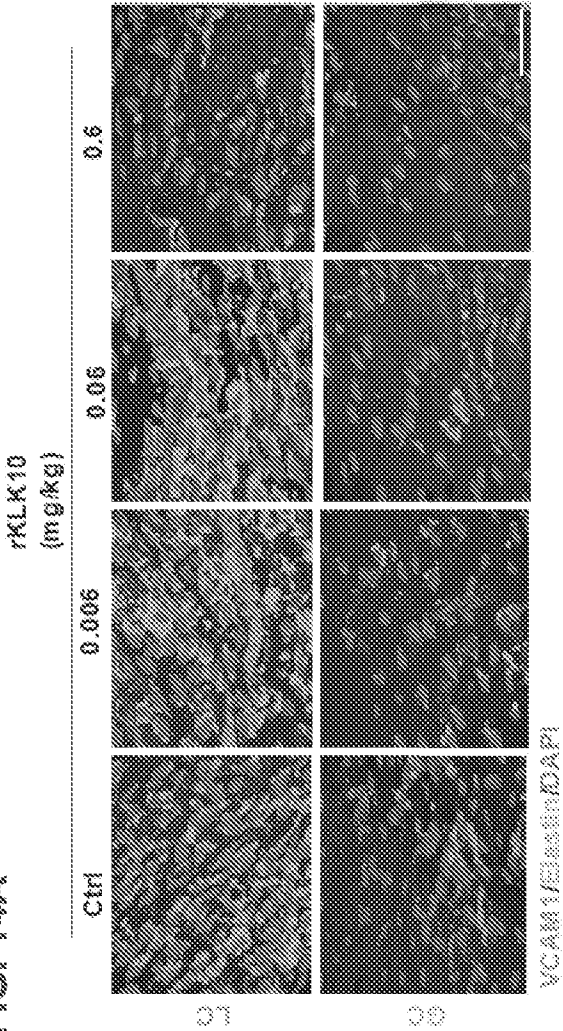


FIG. 14B

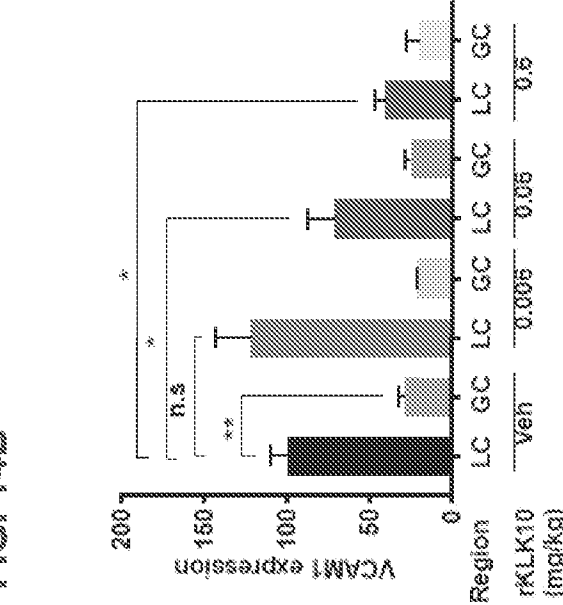


FIG. 15A

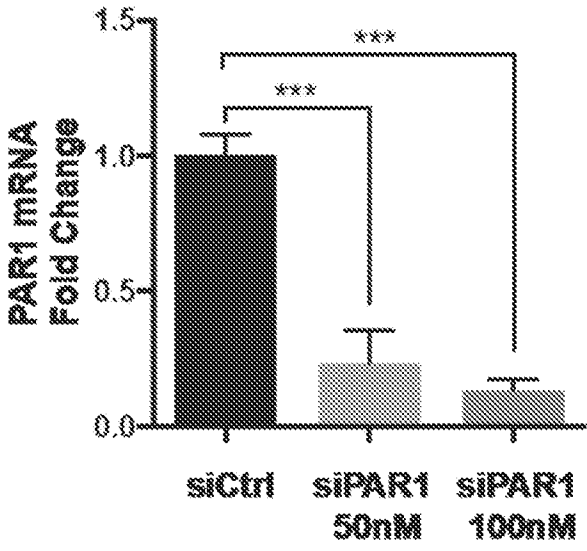


FIG. 15B

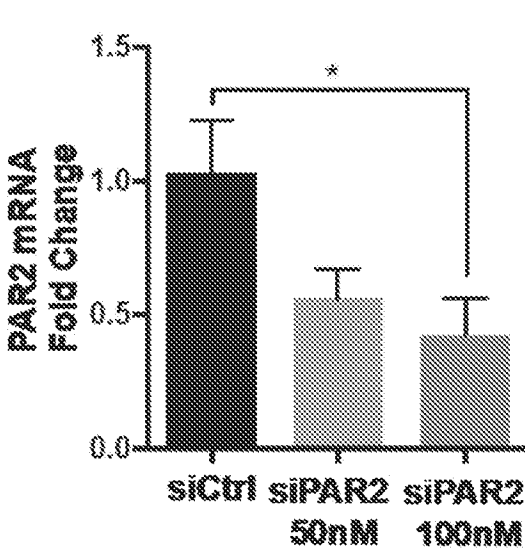


FIG. 15C

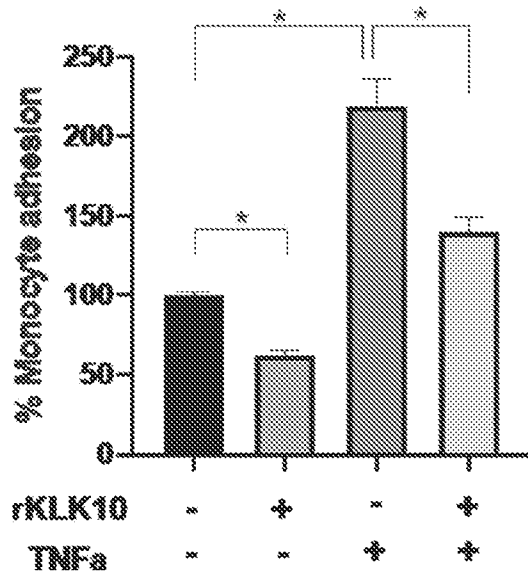


FIG. 18

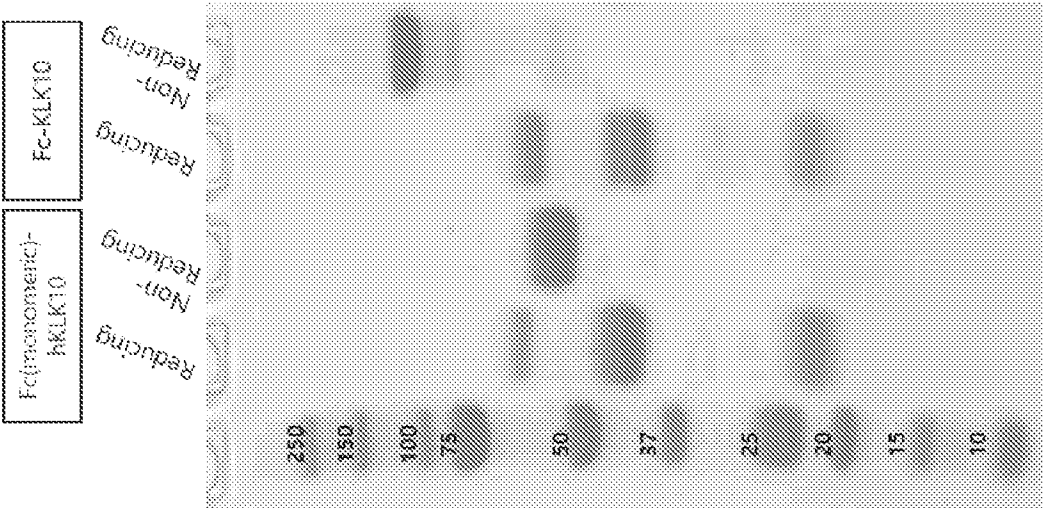


FIG. 17

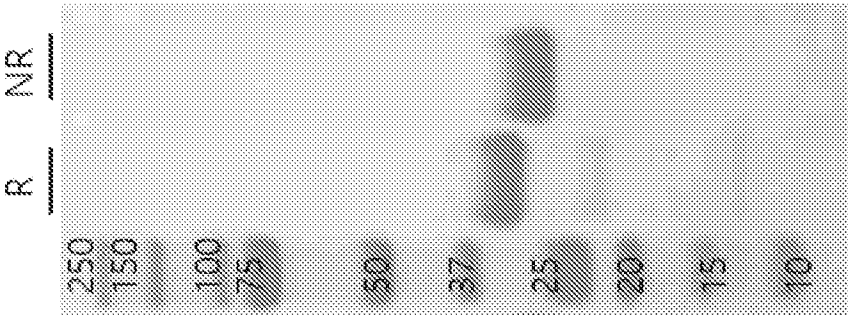


FIG. 16

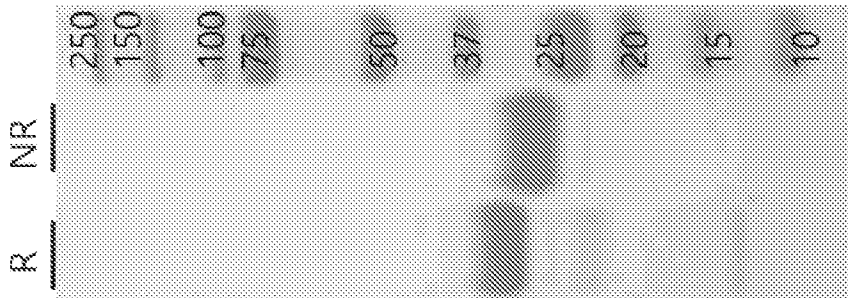
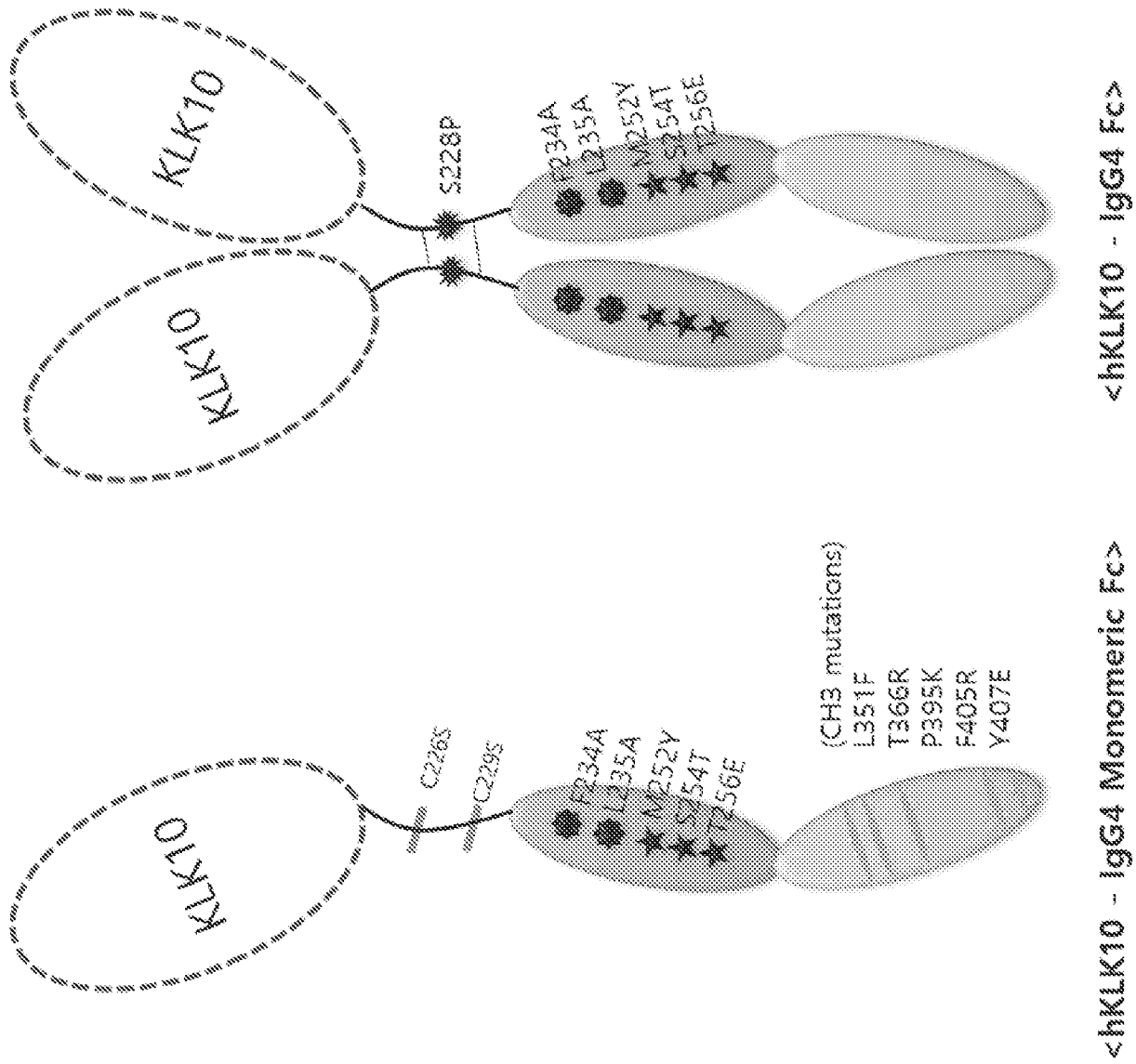


FIG. 19



INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/052524

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/00 A61K47/68 A61P7/12 A61P9/10
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search, WPI Data, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X A	WO 2005/118840 A2 (BAYER HEALTHCARE AG [DE]; GOLZ STEFAN [DE] ET AL.) 15 December 2005 (2005-12-15) p.8 1.22-30, p.19 1.1, p.44 1.9-24, p.59, SEQ ID NO.2 & DATABASE Geneseq [Online] 15 June 2007 (2007-06-15), "Human kallikrein 10 (KLK10).", retrieved from EBI accession no. GSP:AEE60770 Database accession no. AEE60770 the whole document ----- -/-	1,5-8, 25-29, 36,37 2-4, 9-24, 30-35, 38-44



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

7 December 2020

Date of mailing of the international search report

16/12/2020

Name and mailing address of the ISA/

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Authorized officer

Bonello, Steve

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/052524

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
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International application No

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